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Sulfonamide Susceptibility of Stock Strains of Dysentery Bacilli and of Strains from Recent Epidemics.

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(With the assistance of Margaret T. Spencer.)
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The extensive use of sulfonamides in the treatment of bacillary dysentery suggests the need for a suitable method of estimating *in vitro* the susceptibility of shigellae to the bacteriostatic action of these drugs. Most of the reports on the action of sulfonamides on *Shigella* have been concerned with comparisons of the bacteriostatic or bactericidal power of different sulfonamides when tested against a relatively small number of strains. Comparisons of the sensitivity of strains of dysentery bacilli used by different investigators are difficult to draw because there has been an almost complete lack of uniformity in the technical procedures employed. It has been pointed out by MacLeod and Mirick[1] that a desirable test of sulfonamide susceptibility is one "which is relatively simple to perform, clear and easy to read, and [one] with which readily reproducible results can be obtained." The type of culture medium, the number of bacteria in the inoculum, and the time of incubation have been shown by Cooper and Keller[2] to affect the results of tests of sulfonamide susceptibility of dysentery bacilli. In the present study an inhibitor-free medium which permits rapid and heavy growth of most of the strains of dysentery bacilli thus far tested has been devised. The medium and its use in the detection of sulfonamide resistant strains are described.

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[1] MacLeod, C. M., and Mirick, G. S., *J. Bact.*, 1942, **44**, 277.

[2] Cooper, M. L., and Keller, Helen M., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 148.

Cooper and Keller,[3] after reviewing the earlier literature on the subject, presented the results of a study of the susceptibility *in vitro* of *Sh. paradysenteriae* (Flexner) and *Sh. sonnei* to sodium sulfathiazole and sulfaguanidine. In another communication,[4] these authors reported the development *in vitro* of sulfonamide resistant strains of the above types. Hardy[5] reported that cultures of dysentery organisms isolated from human infections which had been treated with sulfonamide for 7 or more days were commonly found to be highly resistant to sulfonamide. He observed that difficulty in treating successfully *Sh. sonnei* infections appeared to be related to the readiness with which this organism develops resistance.

Culture Medium. The indispensability of nicotinic acid, or related compounds, for the growth of dysentery bacilli has been demonstrated by Koser, Dorfman, and Saunders[6] and others, and the requirement for pantothenic acid by certain strains of *Sh. paradysenteriae* (Flexner) has been pointed out by Weil and Black.[7] The medium used in the present study consisted of an acid hydrolysate of casein supplemented with tryptophane, nicotinic acid, pantothenic acid, thiamin, glucose and inorganic salts. Thiamin was included in the medium because a single strain of *Sh. dysenteriae* was encountered which failed to grow in medium to which this vitamin was not added. The growth of a number of other strains of *Shigella* was found to be unaffected by the presence or absence of added thiamin.

The medium was prepared in lots of 4 liters by dissolving in several hundred cc of distilled water, 40 g acid hydrolyzed casein (Casamino Acids Difco), 12 g anhydrous KH_2PO_4, 72 g $Na_2HPO_4 \cdot 12H_2O$, and 8 cc salt mixture. The pH was adjusted to 7.5 with 20% NaOH. After bringing the volume to one liter, the solution was boiled for 10 minutes and filtered through paper. (Sufficient salt mixture for 50 liters of medium contains 22.5 g $MgSO_4 \cdot 7H_2O$, 50 mg $CuSO_4 \cdot 5H_2O$, 50 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $FeCl_3 \cdot 6H_2O$, 20 mg $MnCl_2 \cdot 4H_2O$, 1 cc conc HCl, and distilled H_2O to bring the volume to 100 cc).

To the filtrate was added 160 mg tryptophane, 4 mg nicotinic acid, 4 mg calcium pantothenate and 4 mg thiamin. The volume was brought to 4 liters, the medium distributed in flasks in 500 cc amounts, and then autoclaved. After autoclaving, 5 cc sterile 20% glucose was aseptically added to each flask. If a precipitate formed as a result of autoclaving, the medium was allowed to stand, and the clear supernatant fluid was aseptically transferred to another flask.

Visual examination of the degree of turbidity which developed in inoculated tubes showed that the medium supported rapid and heavy growth of the vast majority of strains tested. No diminution in the growth attained in cultures was observed upon serial subcultivation in the medium. Enumeration, by means of pour plates, of the bacterial populations of 16- to 24-hour cultures of *Sh. ambigua, Sh. sonnei, Sh. paradysenteriae* (Boyd 103), and *Sh. paradysenteriae* (Flexner V) indicated that the cultures contained 240, 400, 480 and 560 million viable organisms per cc respectively.

Technic of Titrations. Stock solutions of sodium sulfadiazine were prepared by dissolving 1 g of drug in 500 cc medium, and sterilizing the resulting solution by Seitz filtration. A series of 2-fold dilutions ranging from M/125 to M/1000 and from M/1250 to M/320,000 was prepared directly in the medium using aseptic technic. Five cc amounts of the dilutions were distributed

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[3] Cooper, M. L., and Keller, Helen M., *J. Pediatrics*, 1943, **22**, 418.

[4] Cooper, M. L., and Keller, Helen M., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 92.

[5] Hardy, A. V., *U. S. Pub. Health Rep.*, 1945, **60**, 1037.

[6] Koser, S. A., Dorfman, A., and Saunders, F., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 391.

[7] Weil, A. J., and Black, J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 24.

TABLE I.
Results of Sulfadiazine Titrations.

Series	Type of Shigella	No. of strains	Lowest concentration of sodium sulfadiazine which completely inhibited growth × 10,000
A	*Sh. paradysenteriae,* Flexner V (I)	8	M/8
	" " " " "	3	M/16
	" " " " "	6	M/32*
	" " " W (II)	1	M/4
	" " " " "	5	M/8
	" " " " "	3	M/16
	" " " " "	6	M/32*
	" " " Z (III)	1	M/2
	" " " " "	4	M/4
	" " " X	1	M/8
	" " " Y	1	M/32*
	" " Boyd 103	1	M/4
	" " " "	8	M/8
	" " " "	3	M/16
	" " " "	1	M/32*
	" " " P119	1	M/8
	" " " "	2	M/16
	" " " "	3	M/32*
	" " " 88	7	M/8
	" " " "	4	M/16
	" " " "	1	M/32*
	" " " 170	1	M/16
	" " " P288	2	M/8
	" " " D1	1	M/8
	" " " "	1	M/16
	" " " P143	1	M/2
	" " " "	3	M/4
	" " " "	1	M/8
	" " " P274	3	M/8
	" " " D19	2	M/8
	" " " "	1	M/32*
	" *sonnei*	3	M/2
	" "	4	M/4
	" "	7	M/8
	" *dysenteriae* (Shiga)	3	M/4
	" "	4	M/8
	" "	1	M/16
	" *ambigua*	7	M/8
	" "	2	M/16
	" *sp. Sachs* Q771	4	M/8
	" " " Q902	2	M/8
	" " " "	1	M/16
	" " " "	2	M/32*
	" " " Q1030	4	M/8
	" " " "	1	M/16
	" " " "	3	M/32*
	" " " Q1167	3	M/4
	" " " "	1	M/8
	" " " Q454	1	M/4
	" *alkalescens*	3	M/4
	" "	3	M/8
	" "	1	M/16
	" *ceylonensis*	9	M/8
	" "	2	M/16
	" "	1	M/32*
	" *madampensis*	1	M/8
B	" *paradysenteriae,* Flexner V (I)	13	M/8
	" " " " "	3	M/16
	" " Boyd 103	1	M/8
	" *sonnei*	7	M/4
	" *ambigua*	1	M/4
	" "	6	M/8

TABLE I. (Continued).

Series	Type of Shigella	No. of strains	Lowest concentration of sodium sulfadiazine which completely inhibited growth × 10,000
C	*Sh. paradysenteriae,* Flexner Z (III)	6	†
	" "	1	M/8
	" "	2	M/16
	" *sonnei*	5	†
	" "	2	M/2
	" "	2	M/4
D	" *paradysenteriae,* Flexner (VIII)	3	†
E	" " " W (II)	3	†
F	" " " VZ (I-III)	4	M/2
IG	" " " W (II)	1	M/2
	Total	219	

* Lowest concentration tested.
† Highest " " was M/125; incomplete inhibition of growth.

among standard 15 mm × 150 mm culture tubes.

In testing the bacteriostatic effect of sulfonamides various authors have shown that the endpoint of the titration is affected by the number of organisms contained in the inoculum. In the present study, preliminary experiments showed that a 10-fold decrease in the number of organisms in the inoculum frequently changed the endpoint of the titration by one tube in the direction of higher dilutions of sulfonamide. This finding served to emphasize the necessity of controlling the size of the inoculum. The inoculum employed was prepared by mixing one standard loopful of an overnight culture, using the medium described, with 20 cc sterile medium. One-tenth cc of the resulting dilution was inoculated into each 5 cc of test medium. The standard loop was made of 24 gauge platinum wire bent in the form of a circle having an inside diameter of 2 mm. The inoculum used represented approximately 1/200,000 cc of culture, and contained of the order of 2500 viable organisms.

The tubes were examined for growth after incubating for 16 to 20 hours at 37°C. The highest dilution of sodium sulfadiazine which completely inhibited growth was chosen as the endpoint of the titration.

Strains of Dysentery Bacilli. Information concerning the original sources of the strains is presented in connection with the results. Grateful acknowledgment is made to Captain C. V. Seastone of the Army Medical Center for supplying us with the cultures of Series A, to Doctor A. V. Hardy of the United States Public Health Service for the cultures of Series B and C, and to Commander L. A. Barnes, H (S), USNR, of the National Naval Medical Center for cultures of Series D, E, and F. Doctor Hardy and Doctor Barnes kindly supplied epidemiological information concerning strains.

Results and Discussion. 230 strains of *Shigella* were tested for their susceptibility to the bacteriostatic action of sodium sulfadiazine. The results of the tests are presented in Table I. It is evident that the strains fall into one of 2 categories in regard to their sensitivity to the sulfonamide: (1) Strains whose growth was inhibited by a drug concentration of M/20,000 or less; (2) strains which grew in the presence of a drug concentration as great as M/125. Of 219 strains, 202 belong to the first category and may be designated as susceptible strains; 17 belong to the second category and may be designated as resistant strains. Eleven strains failed to show optimal growth in control tubes containing no sodium sulfadiazine, and for this reason tests of the susceptibility of these strains have been omitted from the tabulation.

Series A consisted of 159 strains from the culture collection of the Army Medical School. This series contained one or more representative strains of each of the well established species of *Shigella*. It is notable that without exception the strains of this series were of the susceptible variety since growth was inhibited by a concentration of sodium sulfadiazine of M/20,000 or less. Although growth of some of the strains was prevented by a concentration as low as M/320,000 while other strains required as much as M/20,000 for inhibition of growth, there appeared to be little or no correlation between sulfonamide sensitivity and species or type of *Shigella*.

Series B consisted of 31 strains isolated from inmates of an institution in Illinois. With the exception of 2 cases, there was no clinical evidence to suggest the existence of sulfonamide resistance. The 2 exceptions were children who were reported to be intermittently positive for *Sh. sonnei* and *Sh. ambigua* respectively, for a period of several months. Table I shows that all of the strains of this series were susceptible to sodium sulfadiazine.

Series C consisted of 9 strains of *Sh. paradysenteriae* Flexner Z and 9 strains of *Sh. sonnei* all of which were isolated from patients in various institutions in New York. Information which has been supplied to us indicates that the patients failed to respond to sulfonamide therapy. Table I shows that 6 of the *Sh. paradysenteriae* Flexner Z strains and 5 of the *Sh. sonnei* strains were sulfonamide-resistant.

Series D consisted of 3 strains designated as predominantly Flexner VIII. These strains were isolated from patients who failed to respond to sulfadiazine therapy. One of the strains appeared in epidemic form among the personnel of a naval vessel subsequent to sailing from Okinawa. The epidemic is reported to have flared up at a later date at Sagami Bay. All known methods of curbing carriers were said to have been applied without success. The other 2 strains of this series were 2 isolations from an individual who became a carrier following an epidemic on board a naval vessel in the vicinity of Kulambangara, Solomon Islands. The 2 strains were isolated nearly a year after the appearance of the epidemic, and the opinion has been expressed[8] that they do not represent the etiologic agent in the epidemic since 4 other strains from persons on the same vessel were *Sh. paradysenteriae* Flexner W. Table I shows that all 3 strains were sulfonamide-resistant.

Series E consisted of 3 strains of *Sh. paradysenteriae* Flexner W isolated during an outbreak of dysentery which occurred on a naval vessel in the Pacific. The epidemic involved more than 300 men, and there were 2 fatalities. Table I shows that these strains were sulfonamide-resistant.

Series F consisted of 5 strains, 4 of which were isolated during an outbreak on a vessel which had been anchored at a harbor in the Marshall Islands. The fifth strain (*Sh. paradysenteriae* Flexner W) was isolated during the course of an outbreak on a vessel which had sailed from Iwo Jima. Table I shows that all 5 of these strains were sulfonamide-susceptible. Information is lacking concerning the response of the patients to sulfonamide therapy.

Although the present study affords little indication of the precise frequency with which sulfonamide-resistant infections may be expected to be encountered, it nevertheless serves to demonstrate by cultural means the existence of such infections. The occurrence of cases of dysentery failing to respond to sulfonamide and yielding organisms which exhibit *in vitro* a high degree of drug-resistance suggests the desirability of performing sulfonamide-susceptibility tests on a greater scale than has been done hitherto. It would appear that in the future sulfonamide-resistant organisms may constitute a serious threat to the successful treatment of dysentery.

Summary. A method of testing the sensitivity of dysentery bacilli to the bacteriostatic action of sodium sulfadiazine is described. The method involves the use of a medium which is essentially free of sulfonamide inhibitors. Of 219 strains tested, 202 were found to be susceptible to the drug while 17 strains were found to be resistant. Among the susceptible strains were representatives of each of the well established varieties of

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[8] Barnes, L. A., personal communication.

Shigella. The resistant strains included representatives of *Sh. paradysenteriae* (Flexner types W, Z and VZ) and *Sh. sonnei.* Many of the resistant strains were isolated from patients who failed to respond to treatment with sulfonamides. The results serve to emphasize a potential difficulty in the chemotherapy of dysentery.

15312

Comparative Study of Hypertensinase and Proteinase Activity of Blood Plasma.

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Hypertension is inactivated by proteolytic ferments (Muñoz and co-workers).[1] This has been demonstrated by the action of crystalline enzymes. Inactivation of hypertensin with pepsin was accomplished by Alonso, Croxatto and Croxatto,[2] with trypsin and chymotrypsin by Croxatto, Croxatto, Illanes and Salvestrini,[3] and with carboxypeptidase by Plentl and Page.[4] Purified aminopeptidase, obtained from yeast, inactivates hypertensin rapidly. Hypertensinase extracts of kidney and other organs, when examined for their proteinase activity, exhibit characteristics typical of aminopeptidase at a pH near the neutral point (Croxatto and Croxatto).[5] Aminopeptidase, obtained from yeast, and hypertensinase extracts of fresh tissues, red blood cells, blood plasma, etc., show a similar behavior toward oxytocin and vasopressin. Both pituitary hormones are inactivated by the various preparations in the presence of an excess of SH groups added in the form of cysteine or glutathione. The rapidity of their action is due to the hypertensinase activity peculiar to them. (Croxatto and Ebensperger).[6]

It is well known that plasma possesses hypertensinase activity which, though slight, is easy to demonstrate. There are certain indications for the assumption that plasma hypertensinase is of major importance. Even when hypertensin is present in the plasma it cannot enter into the red blood cells. Recent experiments have shown that complete ligation of the arteries of certain parenchymatous organs such as kidneys and liver, has little effect on the pressor response produced by continuous or interrupted injections of hypertensin or renin (Croxatto and Neira).[7] From these considerations, it seemed of interest to investigate the proteolytic properties of plasma as well as its hypertensinase activity under physiologically normal conditions of pH and temperature.

According to Bergmann and his co-workers[8] studies of proteinase specificity can be accomplished quantitatively by using as substrates only a few synthetic polypeptides of known structure, which are hydrolyzed to peptides. Accordingly, we have used the following polypeptides: l-leucyl-glycine, dl-leucyldiglycine, dl-methylleucyldiglycine, carbobenzoxy-glycyl-phenylaline, alpha-benzoyl-

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[1] Muñoz, J. M., Braun Menendez, E., Fasciolo, J. C., and LeLoir, L. F., *Amer. J. Med. Sci.*, 1940, **200**, 608.

[2] Alonso, O., Croxatto, R., and Croxatto, H., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 61.

[3] Croxatto, H., Croxatto, R., Illanes, G., and Salvestrini, H., *Bol. Soc. Biol.*, Santiago, Chile, 1942, **1**, 4.

[4] Plentl, A., and Page, I., *J. Exp. Med.*, 1944, **79**, 205.

[5] Croxatto, R., and Croxatto, H., *Science*, 1942, **96**, 519.

[6] Croxatto, H., and Ebensperger, A., *Bol. Soc. Biol.*, Santiago, Chile, 1944, **2**, 205.

[7] Croxatto, H., and Neira, E., *Bol. Soc. Biol.*, Santiago, Chile, 1944, in press.

[8] Fruton, T. S., Irving, G. W., and Bergmann, M., *J. Biol. Chem.*, 1941, **138**, 249.

TABLE I.
Proteolytic Action of Plasma from Various Species.

Plasma	Amount used cc	Time of incubation hr	Substrate (0.05 M)	Hydrolysis %	pH
Human (A)	2	24	l-leucylglycine	30	7.6
(B)	2	24	”	25	”
(C)	2	22	”	23	”
(D)	2	23	”	24	7.8
(E)	2	24	”	30	”
(F)	2	24	”	47	7.6
Bovine (1126)	0.2	24	”	4	7.5
(1126)	2	22	”	63	7.8
(1159A)	2	24	”	28	7.6
(1159B)	2	25	”	20	7.8
(1154)	2	24	”	34	7.6
(1141)	2	24	”	3	”
Equine (1160A)	2	24	”	46	”
Feline (1869)	2	24	”	85	”
(1869B)	2	24	”	95	”
(1931)	2	24	”	58	”
(1931)	2	24	”	50	”
(1931B)	2	24	”	54	”
Human (A)	2	27	carbobenzoxy-glycyl-l-phenylalanine	—	”
(B)	2	27	”	—	”
Bovine (1126)	2	23	”	40	7.8
(1154)	2	24	”	—	7.6
Feline (1869)	2	24	”	—	”
(1931)	2	24	”	3	”

Benzoyl-l-arginineamide and carbobenzoxy-l-glutamyl-l-tyrosine were not hydrolized by human, bovine and feline plasmas.

l-arginineamide and carbobenzoxy-glutamyl-l-tyrosine, these being substrates for determining the presence of aminopeptidase, carboxypeptidase, trypsinase and pepsinase. An analysis of chymotrypsinase activity had to be omitted for lack of an adequate substrate.

Methods. Arterial blood of cats, cows, and horses, and human venous blood were used. The blood was collected directly in a centrifuge tube containing 3.8% sodium citrate solution in the proportion of 1 to 10. Precautions were taken to avoid hemolysis. The centrifuged plasma was mixed in amounts varying between 0.2 and 2 cc with the various peptides, the hydrolysis of which was measured by the method of Grassman and Heyde,[9] the titration being done 20 to 40 hours after addition of reagents. To prevent troublesome precipitation of albumin from the 2 cc of plasma, the albumin was removed by centrifugation after adding the optimum amount of alcohol required for the titration. The clear liquid was then titrated exactly according to the methods described. In all cases the concentration of substrate was 0.05 M dissolved in a M/15 to M/30 buffered phosphate solution at a pH ranging from 7.3 to 7.8. The free carboxyl groups were determined by adding 0.01 N alcoholic KOH solution. The values obtained in the blank were subtracted. The percentage of hydrolysis was calculated by parallel measurements on mixtures of peptides and amino acids in equimolecular proportions and by using crystallized trypsin and carboxypeptidase with their corresponding substrates. In all experiments sterile material was employed and strictest precautions of asepsis were observed. Frequently the plasma was sterilized by passage through a Seitz filter.

Results. As will be seen in Table I, the estimation of hydrolysis was difficult with the use of minute amounts of plasma, but with a ratio of 2 cc of plasma to 1.36 cc of substrate, an appreciable hydrolysis of l-leucylglycine was obtained within the in-

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[9] Grassmann, W., and von Heyde, W. Z., *Physiol. Chem.*, 1929, **183**, 32.

TABLE II.
Proteolytic Activity of Various Fractions of Plasma Obtained by Precipitation with Ammonium Sulphate.

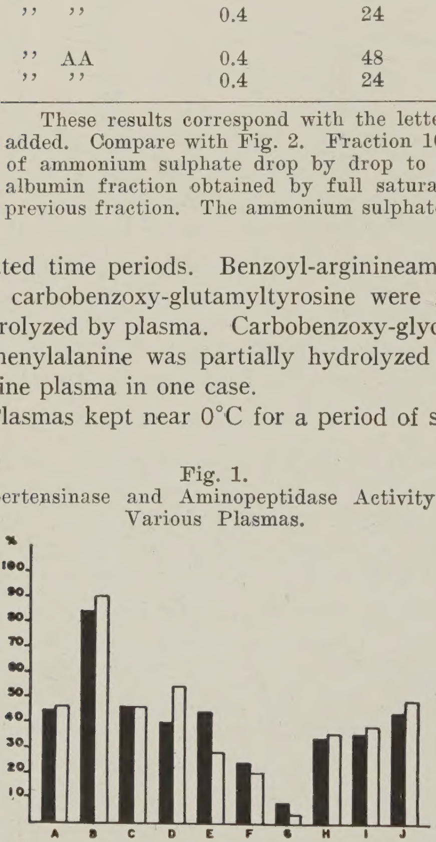
Plasma (fraction)	Amt used cc	Time of incubation hr	Substrate (0.05 M)	Hydrolysis %	pH
1083 AG	0.2	22	l-leucylglycine	28	7.3
,, ,,	0.4	26	,,	63	7.6
,, AA	0.4	26	,,	20	7.4
1154	2.0	24	,,	36	7.6
,, A	2.0	24	,,	38	,,
,, B	2.0	24	,,	49	,,
1083 AG	0.2	22	dl-leucyl-diglycine	24	7.4
,, ,,	0.2	44	,,	56	,,
,, ,,	0.2	48	dl-methyl-leucyl-diglycine	—	,,
,, ,,	0.4	24	carbobenzoxy-glycyl-phenylalanine	25	,,
,, AA	0.4	48	carbobenzoxy-glycyl-1-phenylalanine	3	,,
,, ,,	0.4	24	benzoyl-1-arginineamide	6	,,

These results correspond with the letters H, I, and J on Fig. 1. 3 mg of cysteine were added. Compare with Fig. 2. Fraction 1083AG was obtained by adding a saturated solution of ammonium sulphate drop by drop to half saturation. Fraction 1083AA represented an albumin fraction obtained by full saturation with ammonium sulphate after removing the previous fraction. The ammonium sulphate was removed by dialysis.

dicated time periods. Benzoyl-arginineamide and carbobenzoxy-glutamyltyrosine were not hydrolyzed by plasma. Carbobenzoxy-glycyl-1-phenylalanine was partially hydrolyzed by bovine plasma in one case.

Plasmas kept near 0°C for a period of several weeks retained their ability to split off leucylglycine.

Fig. 1.
Hypertensinase and Aminopeptidase Activity of Various Plasmas.



Black columns: Hypertensinase activity expressed in percentage destruction of the hypertensin; white columns: aminopeptidase activity; A, human plasma F; B, feline plasma 1869B (passed through a Seitz filter); C, equine plasma 1160A; D, feline plasma 1931; E, bovine plasma 1159A; F, bovine plasma 1159B; G, bovine plasma 1151; H, bovine plasma 1154; I, bovine plasma 1154 (fraction A); J, bovine plasma 1154 (fraction B). Fractions A and B were obtained by precipitation with half and complete saturations of ammonium sulphate respectively and subsequent dialysis. Each column represents the average of 3 separate determinations.

The hypertensinase activity of the plasmas bore a close relation to their hydrolyzing effect on l-leucylglycine. In Fig. 1 the % destruction of 2 units of hypertensin by 1 cc of plasma after 2 hours of incubation at 37°, at a pH of 7.4 is shown. The action of aminopeptidase activity was measured in 2 cc of plasma for 1.36 cc of 0.05 M l-leucylglycine at a pH of 7.3 and 7.8 after 24 hours of incubation at 37°.

The treatment of plasma with various concentrations of ammonium sulphate and its subsequent dialysis yields fractions with varying degrees of hydrolytic activity for l-leucylglycine (Table II). The hypertensinase activity of these various fractions closely parallels the hydrolyzing effect of the plasma on l-leucylglycine (Fig. 2). Heating the plasma to 58° and acidifying it to pH 3.2 decreases equally its power to inactivate hypertensin and its aminopeptidase action (Fig. 3).

Discussion. Plentl and Page,[10] by studying the kinetics of the destruction of hypertensin by hypertensinase, found that it fulfilled the characteristics of a first-order reaction. Studies

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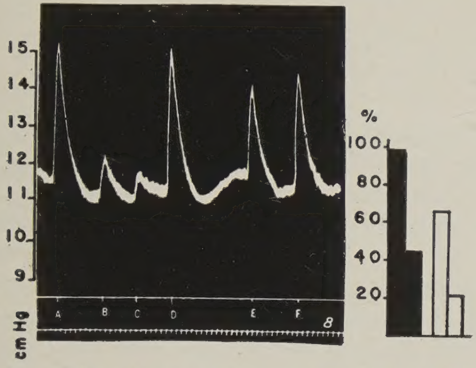
[10] Plentl, A., and Page, I., *J. Exp. Med.*, 1943, **78**, 367.

Fig. 2.
Aminopeptidase and Hypertensinase Activity of Fractions 1083AG and 1083AA. Blood Pressure of a Cat Anesthetized with Dial.

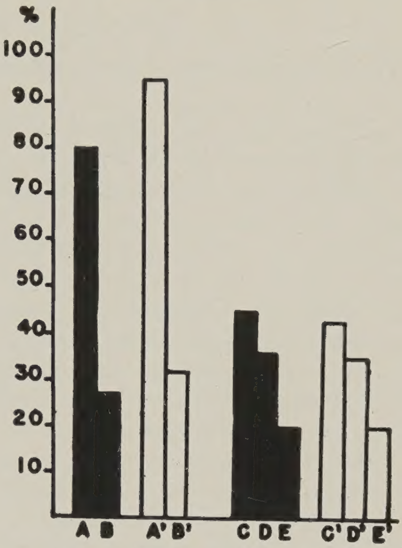


The following mixtures were injected intravenously after 5 hours of incubation at 37° at pH 7.4: At A, 2 units of hypertensin; at B, 2 units of hypertensin plus 0.05 cc of fraction 1083AG; at C, 2 units of hypertensin plus 0.2 cc of fraction 1083AG; at D, 2 units of hypertensin plus 0.05 cc of fraction 1083AA; at E, 2 units of hypertensin plus 0.2 cc of fraction 1083AA; at F, 2 units of hypertensin.

Figure on the right: Black columns: Percentage destruction of hypertensin by fractions 1083AG and 1083AA respectively after 5 hours of incubation. White columns: Percentage break-down of l-leucylglycine after 24 hours of incubation (1.36 cc solution 0.05 mM, pH 7.8) in the presence of 0.1 cc of fractions 1083AG and 1083AA respectively.

on the hydrolysis of l-leucylglycine and on the inactivation of hypertensin by aminopeptidase suggests a similar type of reaction for both (Valenzuela).[11] The magnitudes of both reactions show a close parallelism. These observations and the data obtained on the behavior of aminopeptidase and hypertensinase extracts toward oxytocin and vasopressin strongly support the hypothesis that the hypertensinase activity of plasma is identical with that of aminopeptidase. A systematic investigation is being made of tissue extracts and slices.

Fig. 3.
Hypertensinase and Aminopeptidase Activity of Human and Feline Plasmas, Before and After Heat and Acidification.



Black columns: Hypertensinase activity. White columns: Aminopeptidase activity.

A, percentage destruction of 2 units of hypertensin by 1 cc of feline plasma after 2 hrs incubation; B, same as A, except for previous heating of plasma at 58°, 90 min; A′, percentage break-down of l-leucylglycine (1.36 cc 0.05 mM) by 2 cc of feline plasma after 24 hrs incubation; B′, same as A′, except plasma subjected to same procedure as in B; C, percentage destruction of 2 units hypertensin by 1 cc human plasma after 2 hrs incubation; D, same as C, except for previous heating of plasma to 58°, 60 min; E, same as C, except acidifying the plasma to a pH of 3.2 for 60 min and subsequent neutralization; C′, percentage break-down of l-leucylglycine (1.36 cc 0.05 mM) by 2 cc of human plasma after 24 hrs incubation; D′, same as C′, except subjecting plasma to procedure as in D; E′, same as C′, except subjecting the plasma to the same procedure as in E.

Summary. This study supports the hypothesis that the hypertensinase activity of plasma at a pH of 7.3 to 7.8 and at a temperature of 37° is closely related to its exopeptidase activity, its nature being that of aminopeptidase.

Acknowledgment is made to Prof. M. Bergmann for his generous advice. Some of the peptides used in these studies are being sent to Drs. T. S. Fruton and A. Plentl.

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15313

A Study of Rettger's Bacillus and Related Bacteria.

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Few reports of the occurrence of Rettger's bacillus can be found in the literature prior to its isolation by Cope and Kilander.[1] Originally the organism was isolated by Rettger during his studies of diseases of fowl. It was described, named and classified by Hadley, Elkins and Caldwell,[2] by whom it was considered to be a member of the genus *Shigella*. St. John Brooks and Rhodes[3] studied one strain obtained from Hadley's collection, but aside from noting that it produced indol, they made no mention of its characteristics. The species is assigned to the genus *Shigella* in Bergey's Manual, Vth Edition, where it was originally placed by Bergey and later by Weldin.[4] Neter[5] in reviewing the genus *Shigella* called attention to the scanty and contradictory knowledge of this species. Very shortly, Cope and Kilander in the aforementioned paper rediscovered the species in human gastroenteritis. At about the same time, Stuart and coworkers[6] encountered strains called "type 33111" in their study of the paracolon group.

Rustigian and Stuart[7] in the following year suggested the name *"Proteus entericus"* for these strains. Subsequently these workers[8] secured presumably authentic type strains and demonstrated that the true species name for their "type 33111" and *"Proteus entericus"* was *"rettgeri."* They assigned the species to the genus Proteus. Their conclusions, based on the study of 78 strains are embodied in a recent paper by Rustigian and Stuart.[9]

Fulton and Curtis[10] previously reported studies of this species, and Felsenfeld and Young[11] have isolated strains in the Chicago area. There do not appear to be other published reports of the occurrence of this organism, although its isolation in New England, Michigan, Illinois and Texas suggests that it is of widespread distribution.

Organisms resembling Rettger's bacillus but not identical with it have been isolated from time to time by most students of enteric bacteriology. Such cultures have the cardinal characteristics of the genus *Eberthella*. Fourteen species are recognized by Bergey, most of the descriptions being incomplete. The communications of de Assis[12] and Herring[13] describe certain species of *Eberthella* but there is little published information concerning the characteristics of this group. A detailed search would undoubtedly bring to light many hidden references to isolations of *Eberthella* species, such as have been made by Costa Mandry[14] and Hill *et al.*[15] In a study of 480 fecal specimens from adults,

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[6] Stuart, C. A., Wheeler, K. M., Rustigian, R., and Zimmerman, A., *J. Bact.*, 1943, **45**, 101.

[7] Rustigian, R., and Stuart, C. A., *J. Bact.*, 1943, **45**, 198.

[8] Rustigian, R., and Stuart, C. A., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 241.

[9] Rustigian, R., and Stuart, C. A., *J. Bact.*, 1945, **49**, 419.

[10] Fulton, M., and Curtis, S. F., *J. Bact.*, 1944, **48**, 382.

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[12] de Assis, A., *Bol. Inst. Vital*, Brazil, 1928, **5**, 3.

[13] Herring, L. C., *Am. J. Med. Tech.*, 1942, **8**, 136.

[14] Costa Mandry, O., *Puerto Rico Rev. Publ. Health and Trop. Med.*, 1928, **4**, 212.

[15] Hill, J. H., Seidman, L. R., Stadnichenko, A. M. S., and Ellis, M. G., *J. Bact.*, 1929, **17**, 205.

TABLE I.
Sources of Cultures.

Source	Rettgeri	Eberthellae
Human diarrhea	23	3
” carriers	9	3
” urine	1	1
Monkey	3	3
Rabbit	2	1
Guinea pig	1	0
Hog	1	0
Unknown	4	3
	—	—
Total	44	13

Vaccaro *et al.*[16] isolated 5 species of *Eberthella,* including *belfastiensis, enterica,* and *oedematiens.* The last named species was also observed recently by Fuentes *et al.*[17] and by Neter.[18]

Eberthella strains have been isolated from sources other than the human intestine. Thus Peterson[19] described 3 species found in well water. Duthie and Mitchell[20] found eberthellae in pseudotuberculosis of rodents. Emmel[21] described various eberthellae from parasite-infested fowl.

It is the purpose of the present paper to present a more complete description of Rettger's bacillus than has hitherto been published, and to discuss the relationship between this species and the rarer strains having generic similarities to it.

Observations. The material studied in this work was a collection of 57 strains of gram negative bacilli which were anaerogenic or microaerogenic, motile, and lactose negative. The sources of the cultures are indicated in Table I. The chief feature of this table is its evidence that these bacilli occur in animals as well as in man. The cultures from monkeys were described previously by Preston and Clark[22] and kindly submitted by Preston. The cultures of unknown origin are stock cultures whose source could not be traced.

No less than 44 of these cultures were identified as Rettger's bacillus. The characteristics of this species are fully set forth in Table II. All 44 strains conform quite closely to the reactions set forth although occasionally strains may exhibit one or 2 negative reactions in tests that are regularly positive. Rustigian and Stuart[9] showed that some strains ferment salicin, others do not. In the present series 23 were salicin positive and 21 negative. This was also true for rhamnose which was fermented by 29 strains, not fermented by 15. Occasionally a strain was encountered which failed to conform to the description in one or 2 respects. For example, 3 strains were urea negative, one citrate negative, one glycerol negative, and tests confirmed the absence of motility in a strain received from Stuart although all our own isolations were motile.

Colonies of Rettger's bacillus are rather distinctive on certain plating media. The greenish-brown, mucoid colonies on bismuth sulfite agar are very characteristic. Less so, but still unusual, are the colonies on S-S agar, where brown centers develop which are not at all similar to the intense blackening of *Salmonella* and *Proteus* colonies. No swarming of colonies has been observed on any plating medium, although Rustigian and Stuart[8,9] have reported its occurrence under special conditions.

The motility of this species, originally recorded by Hadley as negative, was demonstrated by Rustigian and Stuart.[8] The difficulty which they appear to have had in demonstrating motility has never occurred in tests using freshly prepared Bacto Motility Test Medium, incubated at 37°C. Bergey describes *Shigella rettgeri* as xylose positive; only one strain of the 44 fermented this pentose. Single strains also fermented cellobiose or maltose or trehalose. Sucrose is fermented after several days when its con-

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[17] Fuentes, C., Angulo, J. J., and Madiedo, O., *Rev. Med. Trop. Parasit.,* 1943, **9**, 22.

[18] Neter, E., *Arch. Path.,* 1939, **28**, 122.

[19] Peterson, O. H., *Thesis, Univ. of Missouri,* 1942.

[20] Duthie, E. C., and Mitchell, Chas. A., *J. Am. Vet. Med. Assn.,* 1931, **31**, 27.

[21] Emmel, M. W., *Florida Ag. Exp. Sta. Bull.,* 1936, 293.

[22] Preston, W. S., and Clark, P. F., *J. Inf. Dis.,* 1938, **63**, 234.

TABLE II.
Characteristics of Rettger's Bacillus.

adonitol	+	levulose	+	xylose	—
arabinose	—	maltose	—	milk	alk
cellobiose	—	mannitol	+	urea	+
dextrin	—	mannose	+	indol	+
dulcitol	—	methylgluc	—	mr	+
galactose	+	raffinose	—	vp	—
glucose	+	rhamnose	+ or —	citrate	+
glucose: gas	—	salicin	+ or —	gelatin	—
1% glycerol	+	starch	—	motility	+
inositol	+	sorbitol	—	sulfide	—
inulin	—	5% sucrose	+	$BiSO_4$: greenish brown	
5% lactose	—	0.5% sucrose	—	DC, SS and Mac plates	+
0.5% lactose	—	trehalose	—	spreading on agar	—

+ : Acid.
— : No acid.
+ or — : Some strains produce acid, some do not.
alk: Alkaline milk.

centration is 5%. At 1/10th this concentration acid is rarely formed. A similar effect of changing the concentration of sucrose on the formation of acid has been observed with *Proteus ammoniae* and *Shigella sonnei.*

The fermentation of adonitol is the most striking single characteristic of this species. In a study of over 1000 strains of Enterobacteriaceae, fermentation of this polyatomic alcohol has been observed only with cultures of Rettger's bacillus, with certain paracolon strains, and occasional strains of uncertain identity. Of all the known species of enteric bacteria, this is the only one characterized by the tetrad of adonitol fermentation, urea hydrolysis, indol formation and growth on Simmons citrate agar. Among these 4 cardinal characteristics, adonitol fermentation is the most restricted in distribution among the other gram-negative bacilli, therefore most nearly a distinctive characteristic of Rettger's bacillus.

Serologically, Rettger's bacillus is distinct from the other Enterobacteriaceae but heterogeneous itself. None of the strains agglutinated in any serum of the Salmonella Typing Center series, nor in any Shigella serum (Lederle), nor in *Proteus,* Morgan's bacillus and columbensis bacillus serums.

Serums were prepared against both formalinized and boiled vaccines of strain 1544 isolated in Detroit. Difficulty was experienced in securing O serum of sufficient titer, the vaccine being quite toxic for rabbits on intravenous injection. The titer of the H serum was 3200, of the O serum 2500. There was little O antibody in the H serum, but the O serum had a higher titer with raw than with boiled antigen. This may indicate a lability of the O antigen which contrasts with the heat stability of this type of antigen in the Salmonella group.

The majority of the cultures, 21 in number, agglutinated in both H and O serums. One agglutinated in O serum but not in H, while 5 agglutinated in H but not in O. Ten did not agglutinate in either serum. Six cultures were too rough to use in agglutination tests. Heating one-half hour at 80°C did not improve the agglutination, and in many instances decreased it. Certain interesting relationships developed during antibody-adsorption experiments. Antigen was prepared from thick tryptose agar plates flooded with one ml of tryptose phosphate broth culture and incubated overnight, suspended in saline and formalinized or boiled. The H serum was diluted 1:50, the O serum 1:5, and 1 ml absorbed twice with the growth from single plates.

Strains 1544 and 571 were agglutinated in the H serum, strains 517 and 519 were not. Complete adsorption occurred with strain 1544, no change with 517 and 519. Removal of agglutinins for 571 without decrease of titer with 1544 resulted when 571 was used as adsorbing antigen. Thus there appear to be strains of Rettger's bacillus having totally unlike H antigens, as well as strains having antigenic fractions in common.

TABLE III.

Name of test	Strains I	II	III	IV	V
adonitol	0	—	—	—	—
arabinose	—	—	+	+	—
cellobiose	—	—	—	—	+
dulcitol	—	—	+	—	—
galactose	+	—	+	+	+
1% glycerol	+	—	—	0	+
inositol	+	+	—	—	+
5% lactose	0	—	+	0	—
0.5% lactose	—	—	—	—	—
maltose	+	+	+	+	—
mannitol	+	—	+	+	—
mannose	+	—	+	+	+
rhamnose	—	—	+	+	—
salicin	+	—	+	—	—
sorbitol	+	—	+	—	—
5% sucrose	0	—	—	0	+
0.5% sucrose	+	—	—	—	+
trehalose	—	+	+	+	+
xylose	—	—	+	—	—
indol	—	+	+	+	+
mr	—	+	—	+	+
vp	+	—	—	—	—
citrate	+	—	—	—	+
gelatin	+	—	—	+	—

+ : Positive reaction.
— : Negative reaction.
0 : Undetermined.

The same 4 strains were used to adsorb the O serum. Only strain 1544 agglutinated, and antibody adsorption was complete in one treatment. Strains 517 and 519 were not agglutinated by the serum and did not adsorb any of the strain 1544 O agglutinins from it. They apparently do not belong to the same O antigenic group as strain 1544, although bacteriologically they are identical with it. Strain 571 slowly but continuously lowered the titer of strain 1544 O agglutinins although it was not itself agglutinated by the serum. It may be that this reaction was also seen by Rustigian and Stuart;[8] they failed to mention whether they observed it with O or H antibodies or with both. This kind of adsorption reaction is by no means unique in this species. There is no generally accepted interpretation of it. It may indicate failure of the second stage of the antigen-antibody reaction, or an antigenic relationship of the partial antigen type between the strains. Certain considerations suggest that it could also result from gradual saturation of the antibody by an antigen fraction liberated from the cells by the heat employed in preparing the O antigen suspension.

It is evident that the serological analysis of Rettger's bacillus has not been completed and that it poses some interesting problems. It is useful to know that the use of the single serum permits ready recognition of most strains, as the agglutination test is the most rapid means of identifying the type.

Strains of the genus Eberthella. Thirteen strains of gram negative bacilli were studied which were motile, anaerogenic, and moderately varied in fermentative powers. Five strains may be Rettger's bacillus but departed rather widely from the typical description and were not agglutinated by "Detroit 1544" H or O serum. Three others form a group which fermented adonitol, glucose, glycerol, levulose, mannose, and sucrose, produced indol, grew on citrate agar and alkalinized milk. One of these cultures was isolated from human diarrhea, one was a stock culture called *Alcaligenes alcalofoetidus* and one was isolated by Preston from a monkey. Although these cultures resembled Rettger's bacillus in their adonitol and indol reactions, their fermentative activity was quite restricted and they were also serologically distinct from that species. The differential reactions of the remaining 5 cultures are listed in Table III. None of these 13 cultures was antigenically related to any *Salmonella, Shigella* or *Proteus* strain studied.

This group of 13 strains is fairly representative of the kinds of organisms included by Bergey in the genus *Eberthella.* Unless great weight is assigned to its ability to hydrolyze urea, Rettger's bacillus would appear to fall within this genus. Rustigian and Stuart[9] have emphasized urea hydrolysis, and as further significant characteristics, mention the occasional production of a small amount of gas together with their observation that swarming occurs under special conditions. There is no clear reason for attempting to include in the genus *Proteus* all bacteria which hydrolyze urea, and the supporting characteristics mentioned are apparently not easy to confirm experimentally. It is desirable to take into consideration all available evidence as to the characteristics and properties of organisms whose systematic relationships are being worked out, even though for purposes of practical rapid recognition a few of the characteristics may be assigned relatively greater weight than the others.

If this view is taken, it is possible to argue that Rettger's bacillus and the other organisms here described belong to the genus *Eberthella.* They are motile, anaerogenic, parasitic, and antigenically distinct from the other Enterobacteriaceae. They have been observed the world over by many workers, although usually they are relatively infrequent. The experience of Cope and Kilander[1] shows that one type at least has occurred with epidemic frequency. The same may happen with any of the other types.

The status of the genus *Eberthella* is at present obscure. It was created originally as a subgenus by Buchanan[23] and elevated to generic rank by Bergey in the first edition of his Manual. At that time Rettger's bacillus was classified as *Eberthella rettgeri.* Subsequently the species was included in the genus *Shigella,* since it had been reported nonmotile by Hadley. Now that its true reactions are known, it is evident that the species never should have been removed from the genus *Eberthella.* Meanwhile, that genus has been left without a type species by the recognition of the fact that *E. typhosa* really belongs in the genus *Salmonella.* In theory, this destroys the genus *Eberthella* as a taxonomic entity. However, there is need for a group designation for these organisms which have in the past been called *Eberthella* species, and the proposed Bacteriological Code does not specifically forbid changing of the type species of a group essentially subgeneric in rank. It is therefore proposed that the genus *Eberthella* be retained as a useful and necessary group, and that *Eberthella rettgeri* (Hadley), the best known and most widely distributed of the species assigned to the genus, be recognized as the type species.

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[23] Buchanan, R. E., *J. Bact.,* 1918, **3**, 27.

15314

Serological Reactions of Levans Formed from Sucrose and Raffinose by Certain Bacilli.*

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Levans, which are water soluble polymers of fructose anhydride synthesized from sucrose and raffinose by some members of the genus *Bacillus* and also by some other bacteria, constitute a chemically well defined class of bacterial polysaccharides.[1] The serological properties of the levans formed by one spore-forming rod (called bacillus N9) and of several strains of *S. salivarius* have been reported.[2,3] The investigation of levans from a larger number of bacteria was required in order to get a more adequate concept of the serological properties of this class of bacterial polysaccharide and thus obtain a better basis for the application of serological methods to problems connected with the general subject of levan formation. Since the most widely distributed levan-forming agents are bacilli, the first step in the accumulation of the required information seemed to be to determine whether or not the levans formed by a number of species or varieties of bacilli had serological properties similar to those of the levans of the previously studied bacillus N9 and *S. salivarius*.

Materials and Methods. The levan-forming bacteria were *B. subtilis* ("Marburg" strain, American Type Culture No. 6051), *B. panis* (American Type Culture No. 8472), 5 strains of *Bacillus* (called A to E, not identified as to species) which had been isolated from feces, air and commercial sugars, and 3 unidentified Gram-positive non-sporulating rod-shaped bacteria† isolated from plants. These 10 bacteria showed features which are commonly associated with levan formation: namely, the supernatant fluids from cultures grown with sucrose or raffinose had a more opalescent or mucoid appearance and a greater content of material precipitable with 2.5 volumes of alcohol than did the fluids of corresponding cultures grown with glucose or other sugars; also the sucrose and raffinose fluids contained considerable amounts of free reducing sugars. The bacteria used as controls comprised the previously studied bacillus N9 and *S. salivarius* S20B from both of which chemically identified levans had been obtained,[2,4] 7 other strains of *Bacillus* (including *B. anthracis*) none of which showed features indicative of levan formation, and *Leuconostoc mesenteroides* B, which was known to produce dextran but no levan;[3,5] the latter was included in order to control the adequacy of the experimental method to distinguish levans from dextrans.

The bacteria were inoculated into 3 sets

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[1] Smith, R. Greig, *Proc. Linnean Soc., New South Wales,* 1901, **26**, 589; Steel, T., *Proc. Linnean Soc., New South Wales,* 1901, **26**, 626. Many later papers are cited in reference 2.

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[3] Hehre, E. J., and Neill, J. M., *J. Exp. Med.,* 1946, **83**, 147.

† Since most, if not all, of the non-sporulating rod-shaped bacteria reported in the literature as levan-formers have been gram-negative varieties, the following description is given of the properties possessed in common by these gram-positive strains. All were aerobic, non-motile, catalase positive, grew readily on ordinary peptone mediums, liquefied gelatin, gave acid from pentoses, grew better at 45°C than at 10°C, and showed no evidence of spore formation in many repeated tests on a wide variety of mediums. The 3 strains, however, differed from each other in fermentation of some carbohydrates.

[4] Niven, C. F., Smiley, K. L., and Sherman, J. M., *J. Biol. Chem.,* 1941, **140**, 105.

[5] Sugg, J. Y., and Hehre, E. J., *J. Immunol.,* 1942, **43**, 119.

TABLE I.

Chemical and Serological Tests Upon Sucrose, Raffinose, and Glucose Culture Fluids.

Kind of bacteria	Sucrose and raffinose cultures						Glucose cultures		
	Poly-fructoside content mg %	Precipitation with antiserums*					Poly-fructo-side content mg %	Precipitation with antiserums	
		Bacillus N9	*Strep. salivarius*	Type 20 pneumococcus†		Type 2 pneumo-coccus		Bacillus N9	All others
				lot 1	lot 2				
B. subtilis	750, 500	3000	3000	3000	0	0	0	10	0
B. panis	800, 300	3000	3000	3000	0	0	0	10	0
Bacillus (strains A, B, C, D, E)	120 to 1000	>1000	>1000	>1000	0	0	0	10	0
Gram-positive non-spore-forming rods (3 strains)	160 to 400	>1000	>1000	>1000	0	0	0	0	0
Controls:									
Bacillus N9	1500, 1300	10,000	10,000	10,000	0	0	0	10	0
S. salivarius S20B	160, 90	3000	3000	3000	0	0	0	0	0
Bacillus (7 non-levan-forming strains)	0	0 or 10	0	0	0	0	0	0 or 10	0
L. mesenteroides B									
sucrose cultures	0	0	0	50,000	50,000	50,000	0	0	0
raffinose cultures	0	0	0	0	0	0	0	0	0

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* Figures indicate highest dilution of the fluid that gave precipitation, 0 indicates no precipitation with 1:10 or higher dilutions.

† Lot 1 of type 20 pneumococcus serum was known from previous tests to react with high dilutions of purified levans as well as with high dilutions of purified dextrans; lot 2 was as highly reactive with dextrans as was lot 1, but did not react at all with levans.

of broth medium containing respectively 0.15 molar concentrations of sucrose, raffinose, or glucose; the base of each medium was tryptose peptone 1%, anhydrous sodium phosphate 0.2%, and sodium chloride 0.5%. After incubation for 10 days at 23°C, the cultures were centrifuged at moderate speed (1500 r.p.m.) for 40 minutes and the supernatant fluids utilized as the test materials.

The presence of levan was determined by analyzing the fluids for polyfructoside content. The method, described previously in detail,[3] consisted essentially of repeated precipitation with alcohol, hydrolysis of the final precipitates in 0.8 N HCl for 15 minutes at 60°C, and analysis of the hydrolysate for fructose by the Roe[6] procedure.

The serological procedure consisted of testing a series of dilutions of the neutralized culture fluids against 3 kinds of antiserums which were known to react with high dilutions (1:500,000 to 1:2 million) of the previously studied purified levans:[2] *viz.*, antiserum of sucrose-grown bacillus N9, antiserum of sucrose-grown *S. salivarius* S20B, and a selected sample (called lot 1) of type 20 antipneumococcus. The use of the 3 kinds of levan-reactive serums made the comparison of the levans in the culture fluids with the previously studied purified levans more complete than would be furnished by tests with a single serum. Although the type 20 serum reacted with dextrans as well as with levans, this complication was controlled by including in all of the tests another sample (called lot 2) of type 20 and a type 2 antipneumococcus serum, both of which were known to be as highly reactive with dextrans as lot 1 of type 20 but entirely non-reactive with levans.

The results are summarized in Table I. In the case of strains listed individually the polyfructoside values are given for both the sucrose and raffinose fluids; in the cases where several strains are listed as a group, the data given are the lowest and the highest values obtained for either the sucrose or raffinose fluids. The serological data for the sucrose and raffinose fluids are combined because no significant difference was observed between them.

Results. The chief point in the data (Table I) is that the sucrose and raffinose culture fluids of *B. subtilis*, *B. panis*, *Bacillus* strains A to E, and 3 non-sporulating Gram-positive rods, which were proved to contain relatively abundant amounts of levan by the polyfructoside analyses, reacted in high dilutions with the same 3 kinds of antiserum with which the previously studied purified levans[2] had reacted. In contrast, the glucose cultures of the same bacteria contained no levan and gave no reactions with the levan-reactive antiserums obtained by immunization with *S. salivarius* and with type 20 pneumococci; slight reactions did occur with the antibacillus serum but these can be dismissed as due to some minor antigen. The chemical proof for the presence of levan in the reactive fluids, the substrate specificity, the serological likeness to the previously studied purified levans, and other factors[‡] make it reasonable to accept the levan nature of the products responsible for the reactions between the antilevan serums and the sucrose and raffinose cultures of the bacilli. The negative reactions of these cultures with the dextran-reactive type 2 and lot 2 of type 20 antiserum serve as a control on the levan reactions obtained from the selected (lot 1) type 20 antiserum. The absence of detectable dextran in the sucrose cultures of the levan-forming bacilli is of some independent interest since levan and dextran occur together in sucrose cultures of certain streptococci.[3,4]

The evidence for the serological similarity of the levans of the 7 sporulating and 3 non-sporulating Gram-positive bacilli to each other and to the levans of the previously studied[2,3] bacillus and *S. salivarius* is based upon their likeness in capacities to react with antiserums obtained by immunization with 3 phylogenetically different bacteria (bacillus, streptococcus, pneumococcus), which is considerably more significant than a likeness in capacity to react with a single antiserum. The high degree of serological similarity found for all the levans studied indicates that similar serological properties are possessed by a large proportion, if not all, of the different members of this class of bacterial polysaccharide. However, since differences in molecular size and detailed chemical structure may occur among them, the levans of a larger number of bacteria will have to be studied before definite conclusions are made on the serological properties of the entire class.

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[6] Roe, J. H., *J. Biol. Chem.*, 1934, **107**, 15.

[‡] The serological reactivity of the levan-containing sucrose and raffinose cultures of the bacilli was destroyed by 15 minutes exposure at 55°C in 0.2 N HCl, which was known[2] to destroy the serological reactivity of the previously studied purified levans. This proof of the lability of the serological properties of the fluids of the bacilli is important because a high degree of susceptibility to mild acid hydrolysis is a well known chemical characteristic of levans.

Summary. Levans formed in sucrose and raffinose broth by *B. subtilis, B. panis,* 5 other strains of *Bacillus,* and 3 strains of nonsporulating Gram-positive rod-shaped bacteria possessed serological properties similar to those previously described for the levans of several strains of *S. salivarius* and of one strain of *Bacillus.* This evidence extends the serological information on the levan class of bacterial polysaccharide and gives a better basis for the application of serological procedures to the detection of bacterial levans both in laboratory culture fluids and in the various materials in which they occur in nature.

15315 P

Relative Efficiency of the Proteins in Polished Rice and in Enriched Wheat Flour.*

BARNETT SURE.

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While a review of the literature discloses that on the same protein intake (5% level) the proteins in whole rice are superior to those in corn and oats,[1] no information was found on the relative biological values of the proteins in polished rice and milled wheat flour, fed on the same protein intake.

During the past year and a half, while engaged in studies on the influence on growth and protein utilization of additions of small amounts of cultured food yeasts to the proteins of cereal flours and cereal grains, it was observed that, on the same protein level in the ration, the proteins in polished rice are far superior to those in enriched white flour. Polished rice fed at an 89% level and enriched wheat flour fed at 57% plane of intake furnished the same 5.8% protein in the ration. The rations contained 2% cellu flour for roughage; 2% cod liver oil and 3% wheat germ oil as sources of fat-soluble vitamins, and a complete salt mixture.[2] Cerelose (dextrose) constituted the rest of the rations. An abundance of the vitamin B complex was furnished daily to each animal separately from the ration. The composition of the vitamin B complex mixture was as follows: 100 μg thiamin, 100 μg riboflavin, 100 μg pyridoxine, 600 μg calcium pantothenate, 100 μg nicotinic acid, 12 mg choline chloride, 12 mg p-aminobenzoic acid, and 3 mg inositol. The protein efficiency is expressed as gains in weight per gram of protein intake. The experimental period was 10 weeks.

Twelve animals on 9% enriched patent wheat flour proteins showed an average gain per animal of 42.2 g and the proteins had an efficiency of 0.88, while on 5.8% wheat

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* Research paper No. 809 Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

[1] Mitchell, H. H., *J. Biol. Chem.*, 1924, **58**, 905.

[2] Sure, B., *J. Nutr.*, 1941, **22**, 499.

flour proteins in the diet, 12 animals made an average gain per animal of 19.3 g and the protein efficiency on this level of intake was 0.72. On the other hand, 30 animals on 5.8% proteins in polished rice made an average gain per animal of 74 g and the efficiency of the rice proteins was 1.86. In other words, on the same plane of protein intake, the animals on the proteins in polished rice gained almost 4 times as much as those on the proteins in enriched wheat flour; also the efficiency of the proteins in polished rice was 158% greater than those in the proteins of the wheat flour.[†]

After the termination of the experiments the animals were sacrificed and the chemical composition of the bodies minus the gastrointestinal contents was determined according to technic previously described.[3] No noteworthy differences were found in the moisture, fat, protein and ash contents of the rice and wheat-fed animals. The results in 2 sets of animals in each group were as follows: Rice-protein fed: Moisture, 56-57%; fat, 18.5-20.6%; protein, 17.3-18.5%; ash, 3.1-3.8%. Wheat-protein fed: Moisture, 57.6-59.4%; fat, 18.0-19.9%; protein, 17.1% and 17.1%; ash, 4.2-4.4%.

[†] Since this manuscript was completed a communication was received from Dr. D. B. Jones of the Food and Nutrition Division of the Department of Human Nutrition, Washington, D. C., in which he states that my results are essentially in agreement with those in progress in his laboratory.

[3] Sure, B., *J. Nutr.*, 1941, **21**, 453.

15316

Potentiating and Pressor Action of Some N-Substituted Hexylamines.

John R. Lewis. (Introduced by A. M. Lands.)

From the Department of Pharmacological Research, Frederick Stearns and Co., Division of Sterling Drug Inc., Detroit, Mich.

Since Frolich and Loewi first observed the potentiation of epinephrine by cocaine in 1910, many other compounds have been found which also increase the pressor response of epinephrine. Gaddum and Kwiatkowski[1] stated that the effect of epinephrine was increased by low but inhibited by high concentrations of ephedrine. They suggested that this might be due to the inhibition of amine-oxidase by ephedrine thus preventing the destruction of epinephrine. Lawrence, Morton and Tainter[2] reported that cocaine, as well as ephedrine and tyramine, blocks the ferment which inactivates epinephrine. Jang[3] studied the effect of 10 sympathomimetic amines on the response of epinephrine and adrenergic stimulation and reported that ephedrine, benzedrine, propadrine, tyramine, cobefrine, sympatol and adrenalone possess the property of sensitizing in small doses, and antagonizing in larger doses, the response of epinephrine or adrenergic stimulation on some or all of the preparations used in their study. Munch and Hartung[4] found the phenylpropanolamine skeleton essential, in their series of arylpropanolamines, for the potentiation of epinephrine. Graham,[5] in an investigation of a series of amines, found that derivatives of phenylethylamine are not as potent as derivatives of phenylisopropylamine in increasing the effects of epinephrine on the cardiovascular system. Jang,[6] in a report

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[1] Gaddum, J. H., and Kwiatkowski, H., *J. Physiol.*, 1938, **94**, 87.

[2] Lawrence, W. S., Morton, M. D., and Tainter, M. L., *J. Pharmacol.*, 1942, **75**, 219.

[3] Jang, C. S., *ibid.*, 1940, **70**, 347.

[4] Munch, J. C., and Hartung, W. H., *J. Am. Pharm. Assn.*, 1930, **19**, 356.

[5] Graham, J. D. P., *Quart. J. Pharm. and Pharmacol.*, 1944, **17**, 19.

[6] Jang, C. S., *J. Pharmacol.*, 1941, **71**, 87.

TABLE I.
Effect on Blood Pressure of N-Substituted Hexylamines, $C_6H_{13}NH$-R.

Compound No.	R	No. of animals	Mean of doses (mg/kg)	Mean of change in blood pressure (mm Hg)
	H	4	0.4 (0.3-0.5) †	+30 (—16, +50) †
185	Methyl	5	0.5 (0.2-1.0)	+55 (—24, +90)
443	Butyl	5	0.9 (0.5-1.0)	—1 (—10, +18)
444	Amyl	4	1.0	+13 (—20, +46)
445	Hexyl	12	0.9 (0.5-1.0)	+19 (—50, +100)
354*	Heptyl	5	0.9 (0.5-1.0)	—6 (—25, +27)
446*	Octyl	4	1.0	+10 (—20, +36)
474	Phenylmethyl (Benzyl)	4	1.0	0 (—20, +36)
475	beta-Phenylethyl	8	1.0	—2 (—30, +30)
476	gamma-Phenylpropyl	4	1.0	0 (—20, +20)
477	delta-Phenylbutyl	4	1.0	+38 (—10, +60)
478*	epsilon-Phenylamyl	4	1.0	+7 (—15, +25)
479*	zeta-Phenylhexyl	5	1.0	—4 (—40, +75)
343	Cyclohexyl	8	1.0	0 (—33, +16)
489	Cyclohexylmethyl	6	0.9 (0.5-1.0)	+30 (—25, +75)
490*	beta-Cyclohexylethyl	9	1.0 (0.5-3.0)	+35 (0,+145)
491*	gamma-Cyclohexylpropyl	5	1.0	+56 (—22, +90)
492*	delta-Cyclohexylbutyl	4	1.0	+23 (0, +42)
493*	epsilon-Cyclohexylamyl	3	1.0	—10 (—75, +30)
494*	zeta-Cyclohexylhexyl	9	1.0	—7 (—40, +18)

* Compounds dissolved in 50% ethanol. Others dissolved in water.
† Range of values.

on the potentiation of epinephrine by ergotoxine, stated that no chemical group is specific for the synergistic or antagonistic action towards epinephrine except that which is essential for sympathomimetic action, and according to Barger and Dale[7] this is an aliphatic amine group. Of the aliphatic amines studied by these workers, n-hexylamine was found to have the greatest vaso-pressor action.

Blicke and Monroe,[8] Blicke and Zienty[9] and Blicke and Data[10] have synthesized, and made available to this laboratory, a series of compounds which can be considered as derivatives of n-hexylamine. While carrying out a routine investigation of these substances, it was noted that some of the amines potentiated the vaso-pressor action of epinephrine and of various other sympathomimetic

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[7] Barger, G., and Dale, H. H., *J. Physiol.*, 1910, **41**, 19.

[8] Blicke, F. F., and Monroe, E., *J. Am. Chem. Soc.*, 1939, **61**, 91.

[9] Blicke, F. F., and Zienty, F. B., *ibid.*, 1939, **61**, 771.

[10] Blicke, F. F., and Data, John B., *Dissertation*, University of Michigan, 1941.

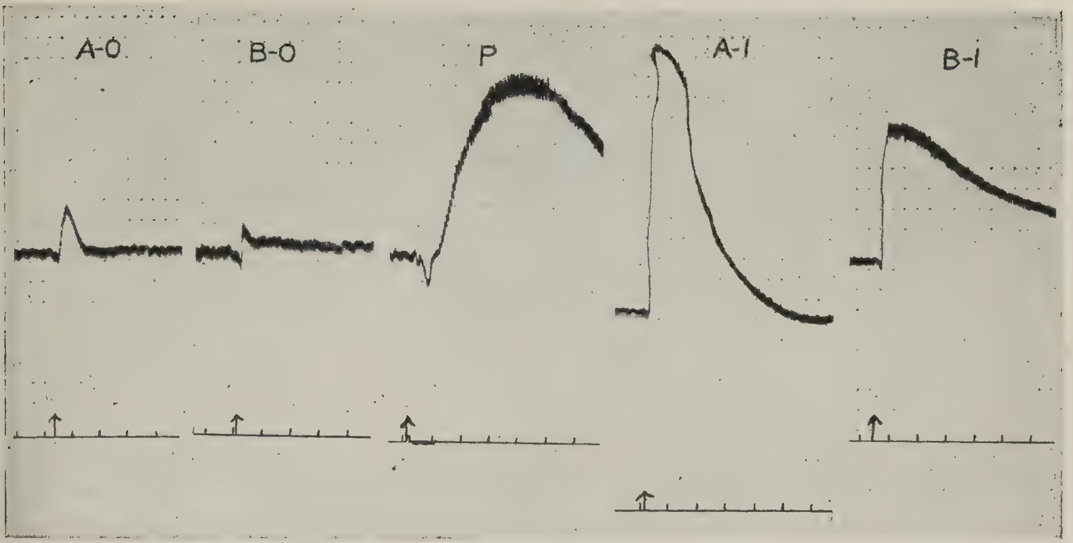

Fig. 1.

Potentiation of pressor amines by other amines. Carotid blood pressure of anesthetized dog. All injections were made into the exposed femoral vein.

A-O: N-methyl-beta-cyclohexylethylamine HCl 0.10 mg/kg before potentiation.
A-1: Same after potentiation.
B-O: Ephedrine HCl 0.05 mg/kg before potentiation.
B-1: Same after potentiation.
P: Hexyl-gamma-cyclohexylpropylamine HCl 1.00 mg/kg.

amines. An investigation of the pressor and potentiating actions of these amines was carried out and the results are reported here.

Experimental. Dogs anesthetized with sodium pentobarbital and atropinized were used as the experimental animals. Blood pressure was recorded from the carotid artery. All injections were made into the exposed femoral vein.

The compounds studied are listed in Table I along with the results indicating their pressor activity. They were dissolved in water or 50% ethanol, as indicated in the table, to make a 1% solution of the hydrochloride salt except in the case of n-hexylamine in which case an aqueous solution of the base was used.

The most active pressor compound in the series is No. 185 (N-methyl-hexylamine). Other compounds which show marked pressor activity are Nos. 445, 477, 489, 490, 491, and 492. Compound No. 491 is approximately one-half as active as N-methylhexylamine on blood pressure. The pressor activity of compound No. 491 is greater than that of its analog, N-methyl-gamma-cyclohexylpropylamine, the pressor action of which was described by Lands, Lewis and Nash.[11] In general, the cyclohexylalkylamines are the most active in this series.

Compounds of the series with groups larger than methyl substituted in the amine group of hexylamine produce a diphasic type of blood pressure response which is characterized by a sharp transient fall followed by a more sustained rise. An example of this is shown in Fig. 1. We believe the fall results from a non-specific depressor action and may be due to a direct relaxing action of the compound on the musculature of the vessels. Tachyphylaxis was observed whenever repeated injections of these compounds were made.

The potentiating effect of a few sympathomimetic amines was determined by comparing the pressor response of the sympathomimetic compound before and after an injection of one of the various hexylamine derivatives. The resulting potentiation is

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[11] Lands, A. M., Lewis, J. R., and Nash, V. L., *J. Pharmacol.*, 1945, **83**, 253.

TABLE II.
Effect of N-Substituted Hexylamines on Pressor Action of Sympathomimetic Amines.

Potentiating compound	Sympathomimetic Amine															
	Epinephrine				N-methyl-beta-cyclohexylethylamine				beta-Phenylethylamine				Ephedrine			
	†A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
185*	4	.8	39	—8	4	.25	37	—5	—	—	—	—	—	—	—	—
443	3	.67	32	+2	2	.1	15	+10	2	.075	22	+17	1	.1	14	+2
444	3	.67	35	+6	1	.1	5	+37	3	.067	21	+21	1	.1	12	+15
445	9	.89	30	+4	9	.12	20	+30	3	.084	19	+40	1	.1	12	+23
354	3	.86	31	+5	3	.1	25	+38	3	.067	21	+45	1	.05	18	+10
446	2	1.0	18	+15	3	.1	24	+32	2	.067	24	+35	1	.05	12	+28
474	3	.86	27	+4	2	.1	13	0	3	.067	31	+2	1	.05	12	0
475	6	1.1	32	+1	3	.13	31	+8	2	.095	34	—2	2	.075	14	0
476	2	.5	25	0	3	.1	20	+32	1	.05	32	0	2	.075	14	+2
477	2	.5	28	+4	3	.13	24	+48	1	.05	32	+12	2	.075	16	+17
478	2	1.0	23	+5	3	.1	23	+26	1	.05	17	+18	1	.05	12	+4
479	4	.9	35	+3	3	.1	22	+24	2	.1	24	+22	2	.075	16	+8
343	5	1.1	32	—1	5	.12	17	+3	2	.075	19	+14	1	.05	10	+8
489	2	.5	39	—4	4	.18	27	+16	2	.05	20	+6	1	.05	12	+3
490	7	1.8	41	+12	5	.14	32	+31	1	.05	12	+20	1	.05	8	+11
491	4	1.1	40	+15	4	.15	28	+42	1	.05	20	+7	2	.05	13	+32
492	3	.67	38	0	2	.15	28	+33	3	.05	21	+27	2	.05	13	+12
493	—	—	—	—	1	.2	19	+41	1	.05	11	+20	1	.05	6	+6
494	5	1.1	34	+20	8	.15	23	+33	3	.05	22	+22	2	.05	11	+6

* Dose of potentiating compound was 1 mg/kg except No. 185 which was .5 mg/kg.

† Column A—No. of animals used.
Column B—Mean dose in mg/kg except epinephrine which is in gamma/kg.
Column C—Average mm rise in blood pressure *before* the potentiating compound.
Column D—Average change in column C *after* the potentiating compound.

given in Table II, and an example is shown in Fig. 1. Since the data represents only a small number of observations in each case the results cannot be compared quantitatively.

The results obtained with epinephrine were much more variable than those obtained with the other sympathomimetic compounds. The inclusion of experiments in which there was no potentiation or even a decrease in the response reduces the positive value of the averages. Compound No. 185 did not produce any sensitization of the pressor responses in the few experiments in which it was used. A sufficient quantity of this compound was not available to carry out further tests. N-methyl-beta-cyclohexylethylamine was not potentiated by No. 474. All other compounds caused an increase in the pressor response of this sympathomimetic amine. The pressor response of beta-phenylethylamine was sensitized by all compounds in the series except Nos. 475 and 476. No specific conclusions can be made concerning the potentiation of ephedrine because of the small number of observations. However, the results obtained appear to parallel those of beta-phenylethylamine.

The pressor activity of the various compounds bears no relationship to their potentiating effect. The duration of the sensitization was found to vary in different experiments from a few minutes to as long as 2 hours. However, in every case it was a reversible phenomenon and the increased pressor responses of the sympathomimetic amines returned to normal, as determined by repeating the injection at various intervals of time. Other sympathomimetic compounds which were observed to be potentiated are: n-hexylamine, tyramine, amphetamine, Sympatol and Privine.

Acute toxicity of a few of these compounds was determined by intraperitoneal injection in albino mice. The results are given in Table III. With the substituted hexylamines, those containing a cyclohexyl ring are more toxic than those containing a phenyl ring. Toxicity is increased by increasing the length of the chain between the ring and the nitrogen. The secondary amine, No. 445 (di-n-

TABLE III.
Acute Toxicity of Some N-Substituted Hexylamines.

Compound No.	Approximate LD 50, mg/kg	Dose (mg/kg), No. of deaths/No. injected
n-Hexylamine	25	15, 0/2; 25, 5/12; 50, 2/2
445	30	15, 1/2; 25, 2/12; 35, 5/5
474	70	40, 0/2; 60, 1/10; 70, 8/10; 80, 7/10; 100, 4/5; 120, 2/2
479	25	15, 2/2; 25, 7/12; 35, 3/5; 50, 10/10
489	50	35, 0/10; 50, 7/10; 75, 2/2
494	<15	15, 10/12; 25, 12/12

hexylamine), was found to be no more toxic than the primary compound, n-hexylamine.

Discussion. Barger and Dale,[7] in their study of the relationship between chemical structure and sympathomimetic activity, found n-hexylamine to be the aliphatic amine having maximum pressor activity. This activity was decreased when the aliphatic chain was lengthened or when there was a substitution in the amino group. Unfortunately we did not have available the N-ethyl- and N-propylhexylamines to complete our series. N-methylhexylamine was found to have the greatest pressor action; di-hexylamine is the next most active amine in the aliphatic series. In view of the previous work, it was somewhat surprising to find pressor activity in an aliphatic secondary amine of this size. The marked pressor response of some of the other compounds such as No. 491, which was most active of the compounds containing a ring, was also not expected inasmuch as it contains a 6-carbon chain substituted on the nitrogen in addition to a 3-carbon chain between the ring and the nitrogen. On the basis of the results obtained with these compounds, it would appear that the pressor activity is primarily a result of the action of the hexylamine portion of the molecule but is modified quantitatively by the other group substituted on the nitrogen.

Since many types of compounds have been reported to potentiate the pressor response of epinephrine, there does not seem to be any chemical group specific for this action. Gaddum and Kwiatkowski[1] have attempted to explain the potentiation of epinephrine by ephedrine with the theory that ephedrine inhibits amine oxidase thus preventing the destruction of epinephrine. This theory of substrate competition has also been applied to explain the pressor action of ephedrine. Ephedrine has been shown not to be destroyed by this enzyme by Blaschko *et al.*[12] The same theory has been applied to the sensitization of epinephrine by cocaine since Lawrence, Morton and Tainter[2] reported that cocaine inhibits amine-oxidase. The pressor response of ephedrine and many other sympathomimetic compounds, including beta-phenylethylamine (Tainter[13]) is diminished by cocaine whereas they are potentiated by compounds in our series. This would indicate that there is more than one mechanism involved in the potentiation phenomenon.

Jang[3] found that cobefrine, which is not attacked by amine-oxidase, was potentiated by ephedrine. We found beta-phenylethylamine, which is destroyed by amine-oxidase and ephedrine which is not destroyed by the enzyme (Beyer and Morrison[14]), both potentiated by hexylamine derivatives. If the pressor action of ephedrine is due to its inhibition of amine-oxidase, then we could not expect it to be potentiated by other sympathomimetic amines whose mechanism of action has been suggested to be the same as ephedrine.

There are a number of enzyme systems which have been recently reviewed by Beyer and Morrison[14] that are capable of inactivating sympathomimetic amines under certain conditions. When one compound increases the action of several sympathomimetic agents which are probably inactivated by different types of enzymes, the theory of substrate competition will not explain the mechanism, unless the potentiating compound were capable of reacting with the various enzymes. This does not seem probable in view of the degree of specificity exhibited by enzyme systems. The effector cell may be sensitized by these potentiators. This raises a fundamental question with regard to the mechanism of action of the various sympathomimetic agents. A completely satisfactory answer has not yet been found. However, it does not seem unlikely that compounds containing long aliphatic chains might alter permeability by surface action and thereby increase the sensitivity of the cell to other compounds.

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[12] Blaschko, H., Richter, D., and Schlossmann, H., *Biochem. J.*, 1937, **31**, 2187.

[13] Tainter, M. L., *Arch. intern. Pharmacodyn.*, 1933, **46**, 192.

[14] Beyer, K. H., and Morrison, H. S., *Ind. and Eng. Chem.*, 1945, **37**, 143.

Summary. 1. A series of 19 N-substituted hexylamines have been studied for their effect on blood pressure and on potentiation of the pressor response of some sympathomimetic compounds. 2. N-methyl-hexylamine was found to have the greatest pressor action with di-hexylamine next most active in the aliphatic series. 3. N-(gamma-cyclohexylpropyl)-hexylamine has the greatest pressor action of those compounds containing a ring. In general, compounds containing a cyclohexyl ring were more active than those containing a phenyl ring or with only aliphatic groups. 4. Many of the compounds in this series have been shown to potentiate the pressor response of epinephrine, beta-phenylethylamine, N-methyl-beta-cyclohexylethylamine, ephedrine and some other sympathomimetic agents. The potentiation of epinephrine was more variable than that of the other sympathomimetic compounds. 5. The possible mechanisms of the potentiation phenomenon are discussed. The amine-oxidase theory of potentiation does not appear to apply to the compounds in this series. 6. Acute toxicity tests indicate that those compounds containing a cyclohexyl ring are more toxic than those with a phenyl ring. Increasing the length of the chain between the ring and the nitrogen increases the toxicity. Di-n-hexylamine is no more toxic than n-hexylamine.

The author is indebted to Mrs. Kathryn Z. Hooper for assistance in carrying out some of the experiments and to Miss V. Loraine Nash for the determination of acute toxicity.

15317

Protective Action of Certain Compounds Against Dietary Hepatic Damage in the Rat.*

JOHN GREEN AND A. BRUNSCHWIG.
From the Department of Surgery, University of Chicago.

In a previous communication[1] experimental studies were reported to show that the protective action of protein (casein) and methionine against chloroform hepatic damage in the dog could be accounted for by the presence of —SH since thioglycollic acid conferred similar protection to the above and glycollic acid, aspartic acid, glutamic acid and sodium thiosulphate did not afford protection. This conclusion was further strengthened by hitherto unpublished experiments in which thiomalic and thiolactic acids conferred protection but malic and lactic acids failed to afford protection. In a second report[2] it was shown that thioglycollic acid conferred protection in the rat against acute hepatic damage by carbontetrachloride but glycollic acid conferred almost an equal degree of protection.

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* These studies were conducted under the Charles and Mary F. S. Worcester Fund. The University of Chicago.

[1] Brunschwig, A., *et al.*, *Arch. Path.*, 1945, **40**, 81.

[2] Brunschwig, A., Johnston, C., and Nichols, S., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 388.

TABLE I.

Summary of Experiments Upon Protective Action of Various Agents Against Parenchymal Necrosis in Liver and of Lipotropic Action.

Supplement to basic diet avg per animal		No. rats	Avg % wt lost	Hepatic cell necrosis series		Fatty infiltration of liver			
						Moderate* series		Severe† series	
				No.	%	No.	%	No.	%
Basal diet only		48	17.7	6	12.5	13	27	15	31.2
Choline	41.8 mg	20	8.6	0		0		0	
Choline Cystine	20 32.3	20	3.2	0		5	25	0	
Methionine	40	18	2.3	0		5	27.8	1	5.5
Thioglycollic acid	24.6	25	30	1	4.0	3	12	10	40.
Glycollic acid	23.3	20	14	1	5.0	7	35	10	50.
Thiomalic acid	31.75	20	18.6	2	10.0	0		19	95.
Malic acid	35.8	19	13.0	2	10.5	5	26.3	9	47.5
Thiolactic acid	28.45	19	15.0	3	15.8	4	21.0	11	58.0
Total No. rats		209							

* ++ fatty infiltration (see text).

† +++ or ++++ fatty infiltration (see text).

Other —SH compounds were also tested but a close correlation between the presence of —SH and protection against hepatic damage was not here observed. Methionine in large doses did not afford protection. The following studies were conducted to determine if certain —SH and other compounds might afford protection against hepatic necrosis of dietary deficiency in the rat and also to observe if such compounds might possess lipotropic action.

Methods. White rats of inbred strain weighing at least 150 g were maintained upon the following diet for 70 days:

	%
Casein (vitamin free)	8
Crisco	38
Glucose	48
Cod liver oil	2
Salt mixture[3]	4

Vitamins added to afford each animal approximately the following daily intake: Thiamine hydrochloride, 75 μg; pantothenic acid, 100 μg; riboflavin, 40 μg; pyridoxine, 65 μg; niacinamid .5 mg. (These were supplied in vitamin B complex-oral, Lederle Lab. Inc.)

This diet has been shown[4] to result in varying degrees of fatty infiltration of the liver, cell necrosis and if maintained for sufficient periods, cirrhosis.

The animals were kept in groups of 8 to 10 in a cage. The daily individual intake of food was not measured but the animals were weighed at weekly intervals and the average of each cage recorded. This served as the indicator of food ingested.

Supplements consisting of the various agents to be tested for protective action on the liver were added to the several portions of the basic diet as indicated in Table I. The quantities of sulphur in the —SH compounds added were equivalent to the S in 40 mg of methionine.

In a few instances the animals died before the expiration of the experiment, the remainder were killed at the termination of the period stated and the livers were fixed in 5% formaldehyde embedded in paraffin and sections stained with hematoxylin and eosin, and in some instances by Mallory's connective tissue stain. Frozen sections for staining with scarlet red were also made in a number of instances.

The features noted were: degree of necrosis (+ to ++++), degree of fatty infiltration (a few scattered vacuolated cells, +; larger groups of vacuolated cells, ++; wide zones of vacuolated cells, +++; practically every cell vacuolated, ++++. The fact that these vacuoles represented fat was controlled by appropriate stains since in marked protein depletion the cytoplasm of hepatic cells often appears vacuolated but does not contain fat). The presence of cirrhosis was also sought for but this was not frequently encountered due in all probability to the duration of the experiments. It was observed only in 3 instances, one in each of the groups receiving glycollic, thiolactic and thiomalic acids respectively.

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[3] Hubbell, R. B., *et al., Am. J. Nutrition*, 1937, **14**, 273.

[4] Gyorgy, P., and Goldblatt, H., *J. Exp. Med.*, 1942, **75**, 355.

The results are summarized in Table I.

Discussion. From the table it is noted that cellular necrosis occurred in 12.5% of the control series and moderate to severe fatty infiltration was present in 58%. Choline afforded absolute protection against necrosis and fatty infiltration. Choline in reduced quantity plus cystine protected against necrosis and also exhibited lipotropic action but not as well as larger doses of choline alone. Methionine exhibited approximately the same effects as choline plus cystine. Thioglycollic and glycollic acids afforded appreciable but not absolute protection against necrosis and *did not exhibit lipotropic action.* Indeed glycollic acid afforded a higher incidence of fatty infiltration than in the control series. Thiomalic, malic and thiolactic acids afforded no protection against necrosis and resulted in a higher incidence of fatty infiltration than in the control series.

The results with thioglycollic and glycollic acids are of interest since by their use it would appear that the lipotropic factor and necrosis protecting factor are shown not to be necessarily identical. For further support of this is the fact that methionine, which has been repeatedly shown to be lipotropic, does not protect against the acute hepatic cell damage caused by carbontetrachloride in rats.

The explanation of the protective action of thioglycollic and glycollic acids against acute hepatic cell damage by carbontetrachloride and by dietary deficiency is not apparent.

Summary. Both thioglycollic and glycollic acids afford a degree of protection against hepatic cell necrosis due to dietary deficiency in rats. These compounds are not lipotropic. The above studies indicate that in certain dietary deficiencies in rats leading to hepatic fatty infiltration and parenchymal necrosis, the factors responsible for both these changes are not necessarily identical.

15318

Experiments with Protoporphyrin.

I. ZELIGMAN. (Introduced by J. G. Hopkins.)

From the Departments of Dermatology and Biochemistry, Columbia University, College of Physicians and Surgeons, and from the Skin and Cancer Unit, New York Post-Graduate Medical School and Hospital, Columbia University.

It has recently been shown[1] that the chromogen of hemoglobin is synthesized *in vivo* from acetate and glycine, but little is known with certainty of the metabolic origin of the porphyrins of the excreta. It has been variously suggested that these are formed (a) during the breakdown of endogenous hemoglobin to bile pigments,[2] (b) by conversion of ingested chlorophyll and hemoglobin[3] and (c) by processes involved in the synthetic production of hemoglobin.[4]

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[1] a. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, 1945, **159**, 45; b. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, 1945, **159**, 567.

[2] a. Schreus, H. T., and Carrié, C., *Klin. Wchnschr.*, 1933, **12**, 745; b. Herold, L., *Arch. f. Gynäk.*, 1934, **158**, 213.

[3] a. Snapper, J., *Arch. f. Verdauungskr.*, 1919, **25**, 230; b. Schumm, O., *Z. f. physiol. Chem.*, 1926, **153**, 225; c. Kämmerer, H., *Dtsches. Arch. f. klin. Med.*, 1924, **145**, 257; d. Vannotti, A., *Porphyrine und Porphyrinkrankheiten*, Berlin, J. Springer, 1937.

TABLE I.
Urinary Excretions of Porphyrins.

Dates 1941	Vol. cc	µg pooled coproporphyrin	Protoporphyrin	Vol. cc	µg pooled coproporphyrin	Protoporphyrin
5/21 to 23	40	7	none	57	7	none
5/23 to 26	80	11	”	84	7	”
5/26 to 28	51	11	”	63	7	”
5/28 to 30	56	7	”	71	5	”
5/30 to 6/2	84	11	”	88	7	”
Injection	solvent only—N/50 NaOH			10 µg/kg protoporphyrin in N/50 NaOH		
6/2 to 4	74	7	none	70	7	none
6/4 to 6	80	11	”	85	7	”
6/6 to 9	90	24	”	94	21	”
6/9 to 11	60	16	”	79	11	”

It has frequently been assumed that coproporphyrin might be derived from protoporphyrin in the body. In perfusion experiments, van den Bergh, Grotepass, and Revers[5] found that liver converted protoporphyrin into coproporphyrin, but Watson[6] and his co-workers were unable to confirm this. The work here reported was designed to bring evidence on this question and also to test the effectiveness of protoporphyrin in photosensitization.

Experimental. Protoporphyrin was prepared by the following method suggested by Dr. E. H. Maechling.[7]

To human clotted blood, was added an equal volume of concentrated HCl and the mixture was heated over a steambath for an hour till all clots were completely broken up. The dark red mixture showed absorption bands at 667-640 mµ, 602-593 mµ, 588-546 mµ: This was cooled and distilled water added—3 parts water to one part blood mixture. The diluted mixture was twice extracted with 1½ volumes chloroform. The acid chloroform extract was dichroic (greenish-pink), had a red fluorescence and showed absorption bands at 612-604 mµ, 587-583 mµ, and 568-554 mµ. The emulsion formed at this point was broken up by shifting the mixture from one separatory funnel to another and allowing it to separate for 20 to 40 minutes. The chloroform solution was washed with portions of water until the washings were neutral to litmus. The neutral chloroform extract showed absorption at 634-628 mµ, 590-577 mµ, and 547-536 mµ. As the acid was removed, the chloroform solution turned from a dichroic greenish-red to a red color. The neutral chloroform solution was extracted with 1/6 volume N/20 NaOH. The dark brown alkaline solution had absorption bands at 638-628 mµ, 585-573 mµ, and 557-543 mµ. Twenty-five cc glacial acetic acid were added. This was then extracted with 200 cc ether. The acid layer was washed with distilled water until washings were neutral to Congo Red. The ethereal solution was then filtered. The pink neutral ethereal extract had an intense red fluorescence and strong absorption bands at 637-630 mµ, 606-581 mµ, 577 - 574 mµ, 542 - 531 mµ, 512 - 499 mµ. The ether was evaporated off; the resulting amorphous residue was virtually pure protoporphyin, as indicated by solubility and spectral characteristics.

Various dilutions of the protoporphyrin in 16 cc ether were made and studied spectroscopically in a 200 mm polariscope tube. The end-points of spectral band detectability

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[4] a. Rimington, C., *Onderstepoort J. Vet. Sci. and Animals Ind.*, 1936, **7**, 567; b. Dobriner, K., *J. Biol. Chem.*, 1937, **120**, 115; Borst, M. u., and Königsdörffer, H., *Untersuchungen uber Porphyrie mit besonderer Berücksichtigung den Porphyria congenita*, Leipzig, S. Hirzel, 1929; d. Dobriner, K., and Rhoads, C. P., *Phys. Rev.*, 1940, **20**, 416.

[5] Van den Bergh, A. A. H., Grotepass, W. u., and Revers, F. E., *Klin. Wchnschr.*, 1932, **11**, 534.

[6] a. Watson, C. J., Pass, I. J., and Schwartz, S., *J. Biol. Chem.*, 1941, **139**, 583; b. Salzburg, P., and Watson, C. J., *J. Biol. Chem.*, 1941, **139**, 593.

[7] Personal communication.

TABLE II.
Urinary and Fecal Excretion of Porphyrins.

Dates 1941	Vol. cc	μg urine coproporphyrin	μg stool coproporphyrin	μg stool protoporphyrin	Vol. cc	μg urine coproporphyrin	μg stool coproporphyrin	μg stool protoporphyrin
6/13 to 16	80	11	43	41 ?	70	11	61	58 ?
6/16 to 18	170	8	29	147	74	7	47	247
6/18 to 20	65	11	89	454	65	14	33	363
6/20 to 23	105	24	99	517	90	24	55	265
Injection		solvent 10 cc N/50 NaOH/kg				protoporphyrin 10 mg in 10 cc N/50 NaOH/kg		
6/23 to 25	75	8	116	606	45	7	42	1266
6/25 to 27	81	7	89	545	70	11		1764
6/27 to 30	125	34	56	487	110	36	98	942
6/30 to 7/2	105	14	34	337	75	32	78	1184

were observed with 4 γ at 633, 12 γ at 576 mμ, and 20 γ at 606 mμ. It had previously been observed that hematoporphyrin and coproporphyrin were spectroscopically indistinguishable. Their corresponding end-points of detectability were observed with 3 γ at 623 mμ, 7 γ at 567 mμ, and 14 γ at 596 mμ.

Quantitative estimation of the amount of porphyrin with this spectroscopic method was based on Lageder's[8] method as employed by Kapp and Coburn[9] and by Maechling.[10] The spectroscopic examination was made with a Bausch and Lomb wave-length spectrometer No. 628. The light source was a (Burn Base Down) Projection bulb—Mazda G. E. 6V, 18A.

Animal Studies. Eight white rats weighing about 200 g each, were kept, 4 in each of 2 metabolism cages, on vitamin D free[11] Rockland rat diet. The pooled urine from each cage was collected every 48-72 hours and the urine extracted for proto- and for coproporphyrin, as follows:

Urine was treated with 3 cc of saturated sodium acetate solution and ⅓ volume of glacial acetic acid and extracted twice with equal volumes of ether. The combined ethereal solutions were washed twice with ⅕ volume dilute sodium acetate solution (approximately 10 cc saturated sodium acetate solution to 600 cc distilled water) and extracted 3 times with 10 cc 5% HCl. The combined acid extracts were neutralized to Congo Red with saturated sodium acetate solution, and 10 cc of glacial acetic acid were added. The solution was extracted twice with equal volumes of ether and the combined ethereal solution was washed twice with dilute sodium acetate solution and shaken 3 times with 10 cc portions of 0.5% HCl. The combined acid solution was washed with an equal volume of chloroform, neutralized to Congo Red with saturated sodium acetate solution, and treated with 2 cc of glacial acetic acid. The acidified solution was extracted twice with 10 cc of ether and the combined ethereal solution containing coproporphyrin examined spectroscopically by the absorption band end-point method described.

The chloroform solution was extracted twice with 10 cc of N/20 NaOH; the combined alkaline solution acidified with glacial acetic acid and extracted twice with 10 cc of ether. The ethereal solutions were combined and examined spectroscopically by the dilution method for protoporphyrin.

After the approximately basal level of coproporphyrin output had been determined, the 4 animals in one cage were injected subcutaneously with protoporphyrin dissolved in N/50 NaOH at a level of 50 mg per kg body weight. The 4 animals in the other cage received an equal subcutaneous injection of the solvent alone. No striking difference in the urinary output of coproporphyrin between the 2 cages was noted. No protoporphyrin was excreted in the urine by either set of animals. (See Table I).

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[8] Lageder, K., *Arch. f. Verdauungskr.*, 1934, **56**, 237.

[9] Kapp, E. M., and Coburn, A. F., *Brit. J. Exp. Path.*, 1936, **17**, 255.

[10] Maechling, E. H., *J. Lab. and Clin. Med.*, 1941, **26**, 1676.

[11] Slanetz, C. A., *Am. J. Vet. Research*, 1943, **4**, 182.

In order to rule out the possibility that the protoporphyrin or the converted coproporphyrin was excreted via the stools, a similar experiment was performed, both urine and stool being collected. The urine was collected, extracted and tested quantitatively as in the previous experiment. The stools, collected for 48-72 hours from each cage, were pulverized and shaken up and extracted 3 times with 200 cc glacial acetic acid. The acetic acid solutions were combined. A ⅓ aliquot portion was extracted twice with equal volumes of ether and the ethereal solutions combined. The remainder of the extraction and quantitative estimation was carried out exactly as outlined for the urine. (See Table II).

Following the injections, the protoporphyrinized animals excreted large amounts of protoporphyrin as such in the feces, but there was no increase of coproporphyrin excretion in either urine or feces.

Photosensitization Experiments. Three weeks after the injections, exposure of the 8 animals to sunlight from 9:30 a.m. to 4:00 p.m. caused the death of 3 protoporphyrin-injected rats on the first afternoon and one control animal the next morning. Autopsy showed that the animals still had considerable pigment at the sites of injection. These pigmented tissues were removed and placed in alcohol which then showed the typical spectrum of protoporphyrin.

Similar white rats on the same diet were used in other photosensitization experiments. Following injection of protoporphyrin 10 mg/kg subcutaneously into some of the animals and of the same amount of the solvent, N/50 NaOH, into control animals, the animals were exposed to a carbon arc lamp and to sunlight with and without a window-glass filter. No striking difference between the control and experimental animals was noted.

Summary. 1. A simple method for the preparation of protoporphyrin from blood is described. 2. White rats were injected subcutaneously with protoporphyrin and were found to excrete greatly increased amounts of protoporphyrin in the feces. No urinary protoporphyrin nor increase of urinary coproporphyrin excretion following the injection was noted. Much of the injected protoporphyrin remained at the site of injection. There was no evidence that the rat is able to convert injected protoporphyrin to coproporphyrin. 3. Photosensitization experiments of protoporphyrin-injected white rats to carbon-arc and sunlight were unsuccessful.

15319 P

Early Changes Induced in Purkinje Cells of Rabbit by Single Massive Doses of Roentgen Rays.*

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That nerve tissue is highly resistant to Roentgen radiation is well known through reports in the literature indicating that large repetitive doses will induce pathological changes in the nerve cells both in man[1] and in laboratory animals.[2,3] Time correlation of

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Wachowski, T. J., and Chenault, H., *Radiol.*, 1945, **45**, 227.

[2] Davidoff, L. M., Dyke, C. G., Elsberg, C. A., and Tarlov, I. M., *Radiol.*, 1938, **31**, 451.

[3] Lyman, R. S., Kupalov, P. S., and Scholz, W., *Arch. Neurol. and Psychiat.*, 1933, **29**, 56.

TABLE I.

Rabbit	Dose	Survival
330	1000 r (220 Kv, ¼ mm Cu)	16 hr
331	" "	24 "
332	" "	48 "
333	" "	4 days
334	" "	9 "
335	" "	69 "
347	3000 r "	died during exposure
348	" "	" following "
349	" "	18 hr
350	" "	43 "
351	" "	8 days
352	" "	13 "
401	" "	0 hr
402	" "	still alive
403	" "	5½ hr
404	" "	12 hr
405	" "	18 "
406	" "	still alive
407	3500 r (140 Kv, no filter)	0 hr
408	4000 r (220 Kv, ¼ mm Cu)	0 "
409	" "	3 "
410	" "	13 "
411	" "	still alive
412	" "	" "
400	" "	" "

these changes has not been made in most of the reported studies. The present investigations were designed to clarify this matter by utilizing single massive exposures which serve as a starting point in relating histological change with elapsed time.

Twenty-five rabbits, 4 to 5 lb in weight, were irradiated in 4 groups of 6 and one singly. With the aid of a special holder, the heads of the animals were held in place in a circle of small radius and simultaneously irradiated. The dosage is listed in Table I.

The changes observed in the treated animals were chromatolysis, vacuolization of the cytoplasm, pyknosis, and loss of cells through neuronophagia. The animals killed immediately following irradiation showed marked lysis of the Nissl material of the cells. This chromatolysis is short-lived, being but slight in No. 349 which was killed at 18 hours and not to be noticed at all in the first member of the 1000 r series. Rabbit No. 408, killed immediately after irradiation shows cells which demonstrate not only complete breaking up of the Nissl bodies into a fine lightly staining powder, but also reveal the appearance at the periphery of the cell of a wide margin of clear cytoplasm. Neighboring cells show large clear vacuoles occupying this marginal zone and in some instances replacing entirely the unstained cytoplasm. Further studies on the relationship of the chromatolysis, the appearance of the clear margin of cytoplasm, and the vacuolization are being made.

Pyknosis of some cells is seen in the early members of the series and becomes more marked in those killed later. Neuronophagia is not observed where less than 24 hours had elapsed following irradiation. It is severe in all of the later instances.

There is a parallelism between the cellular pathology described above and the observed symptomatic behavior of the animals. Those receiving the heavy doses were observed to be apathetic, somnolent, and even prostrate immediately thereafter. All showed dyspnea and slight or marked exophthalmos. Rapid clearing up of these conditions followed the termination of the irradiation. In one-half hour marked improvement was seen and 12 hours later the survivors showed only slight signs. Conjunctivitis developed during the first 12 hours and lasted as long as 3 or 4 days. Hyperexcitability and excessive saliva-

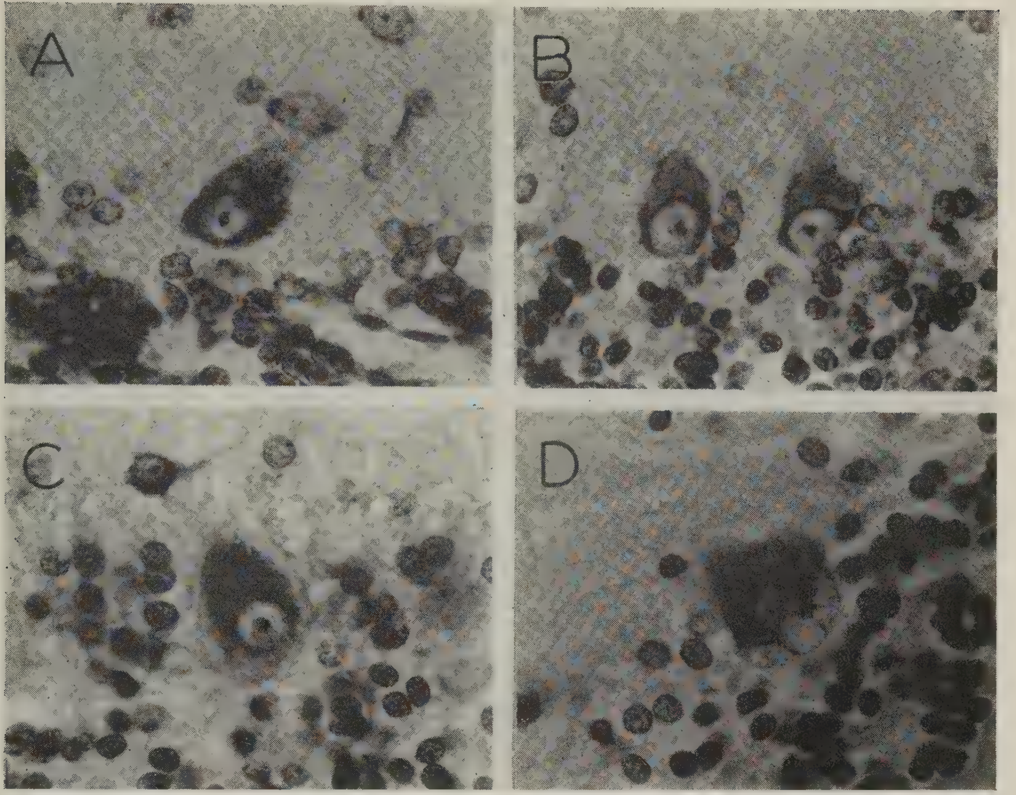

Fig. 1.
a. Normal Purkinje cell from control preparation.
b. Chromatolyzed cells.
c. Cell showing clear margin of cytoplasm.
d. Cell showing vacuolization of margin. × 600.

tion developed in a week or 10 days.

Immediate and severe chromatolysis produced by irradiation is in marked contrast to the more familiar axon reaction. Changes concomitant with chromatolysis and apparently related to it, the formation of a clear peripheral zone of cytoplasm and the appearance of vacuoles, appear only as immediate effects. Temporally these are related to certain symptoms which are also fugitive. In several days the whole syndrome is replaced by the well-known degenerative picture.

15320

Time Course of Changes in Sensory Cells of Trigeminal Ganglion of the Rabbit Following Irradiation.*

Rosalind Novick. (Introduced by Berry Campbell.)
From the Department of Anatomy, University of Minnesota.

Immediate and delayed effects of Roentgen ray exposure upon neurons may be separated by analysis of the time course of chromatolysis induced. To achieve this end it is essential that single effective doses be employed

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

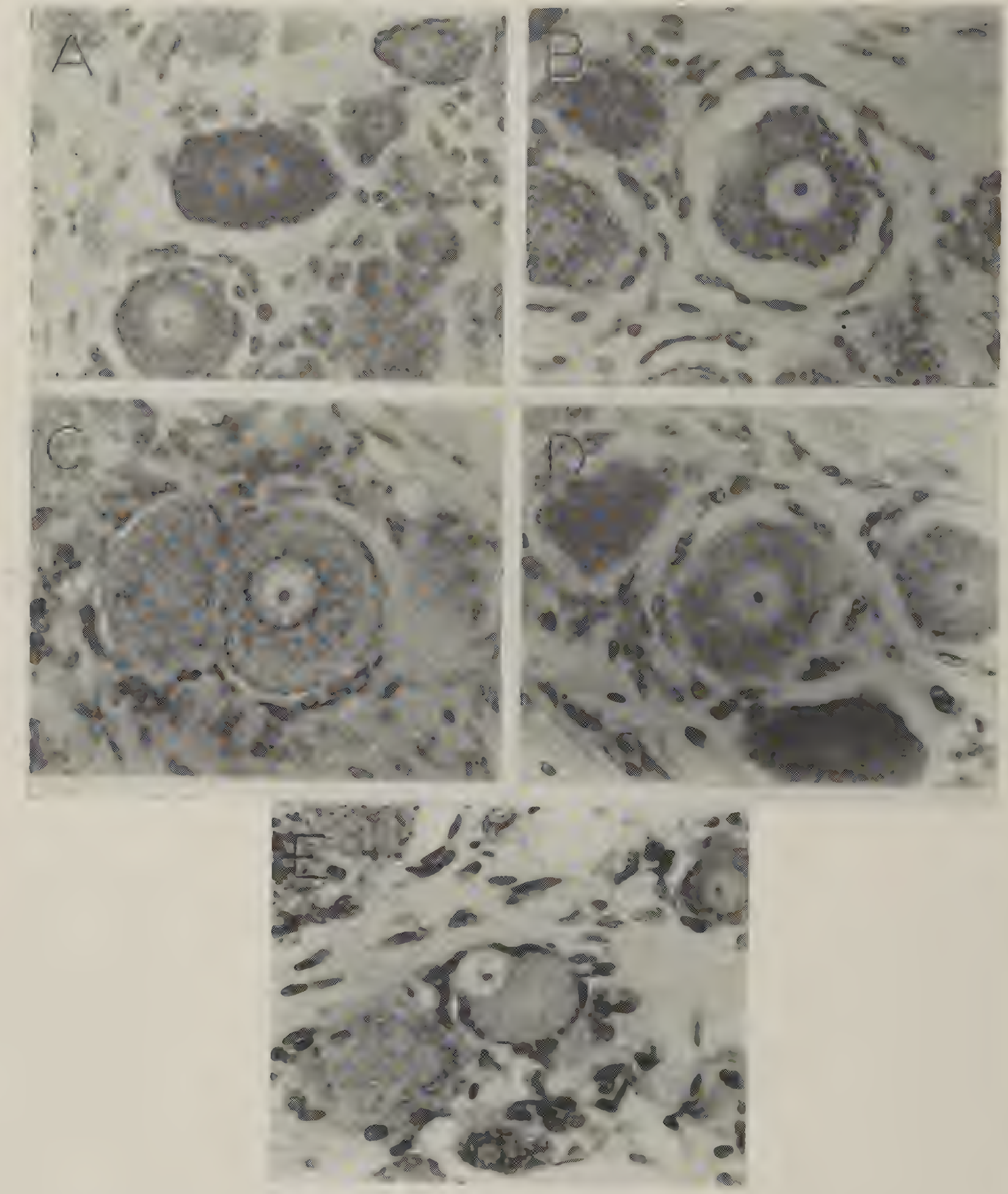

Fig. 1.

a. Sensory cell of trigeminal ganglion showing hyperchromatism.
b. Normal sensory cell.
c. Cell showing mild chromatolysis.
d. Cell showing severe chromatolysis.
e. Cell showing eccentric nucleus, severe chromatolysis. × 400.

in order that a starting point in time for such correlation be determined. As the literature on the effects of irradiation on the nervous system has not included experiments incorporating these conditions, a study applying the quantitative method of Campbell and Novick[1] was made of the sensory cells of the trigeminal ganglion of the rabbits re-

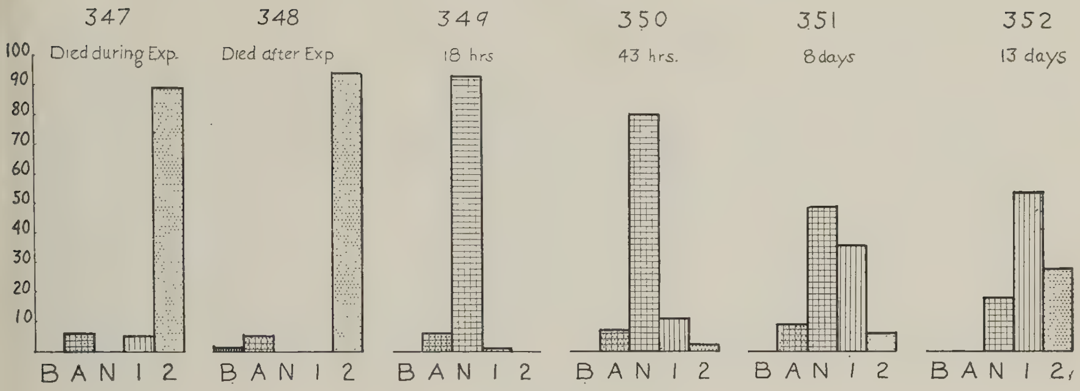

Fig. 2.
Frequency polygons illustrating differential cell counts of irradiated ganglia.

ported in the foregoing paper.[2] For histological methods and experimental conditions reference may be made to that paper.

The trigeminal ganglia of 20 irradiated animals, which had received single doses of 1000-4000 Roentgen units to the head were examined to determine the changes in Nissl pattern as a result of irradiation. In the second series of rabbits, animals 447-452, which had received 3000 Roentgen units, 100 successive cells from each ganglion were counted and classified into the groups described below. Although all the neurons were observed to undergo comparable changes, because of the difficulty of evaluating severity of chromatolysis in small cells, counts were made only of cells 33 μ in diameter or larger.

The establishment of a chromatolytic index for the sensory cells of the trigeminal ganglion required the separation of the following stages:

B—Pyknosis: The cell is shrunken, angular in shape, so darkly staining as to obscure the condition of the nucleus and cell contents.

A—Hyperchromatism: The cell is round, the Nissl particles larger, more heavily staining than normal. This stage is illustrated in Fig. 1a.

N—Normal: The cell is round. The Nissl particles may vary widely in size from cell to cell but are always discrete and are uniform in size within any specific cell. Fig. 1b.

1—Mild chromatolysis: The Nissl particles are dustlike in some parts of the cell but the normal pattern may still be observed in the perinuclear region. Fig. 1c.

2—Severe chromatolysis: The Nissl particles are dustlike throughout the cell. In some cases the nucleus is displaced to a peripheral position and only a dark margin of blue-staining material is visible. Fig. 1d.

A series of polygons illustrating the conditions existing in each rabbit of the second series clarifies the nature of the response of trigeminal cells. Fig. 2. The reaction of these cells may be classified into 3 distinct phases of injury. Initially, severe and extensive chromatolysis immediately follows irradiation. 89-94% of the ganglion cells are completely chromatolyzed. Not a normal cell may be found in the ganglion. This condition was observed in all series. As soon as exposure to irradiation is ended, however, a period of recovery sets in, so that as early as 3 hours after the end of the irradiation period, marked improvement can be noted, and by the end of 18 hours, the ganglion cells have returned to an apparently normal condition. At 43 hours the beginning of a second wave of chromatolysis is clearly observable. The number of chromatolyzed cells and the severity of chromatolysis increases steadily until 82% of the large ganglion cells are affected at 13 days. The second chromatolytic wave is sharply separable from that occurring during the irradiation period. First, the onset

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[1] Campbell, B., and Novick, R., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 425.

[2] Campbell, B., Peterson, S. C., and Novick, R., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 353.

is delayed until 43 hours after irradiation, and is preceded by a normal condition. Second, the onset is not simultaneous in each cell of the ganglion. The chromatolysis having once begun, progresses slowly. Initially it usually appears at a point halfway between the nucleus and the cell membrane and gradually spreads both centrally and peripherally, eventually involving the entire cell body. Some cells are so severely affected that their nuclei are displaced to a peripheral position and the Nissl substance entirely disappears, leaving only a blue-staining border, Fig. 1e. Neuronophagia of dead ganglion cells may be observed in animals surviving for as long as 13 days. The second wave of chromatolysis is sufficiently persistent as to be discernible even after as long as 69 days.

The parallelism between the condition of the trigeminal ganglion cells and the clinical picture presented by the rabbits is marked. At the end of the exposure the cells were severely damaged. At this time the rabbits appeared acutely ill, displaying dyspnea, exophthalmos, somnolence and postural abnormalities. They improved very rapidly, however, and after 12 hours seemed almost normal. Simultaneously, the cells underwent rapid recovery. The second chromatolytic wave was paralleled by a second phase of illness in the animals. They became emaciated and salivated excessively.

Discussion. In an investigation of radiation effects on the dorsal root ganglia of rats,[3] Ma and Hsü concluded that the effects of Roentgen rays were indirect only, and that the pathological condition of these cells was caused by a disturbance in the nutrition of the cells as a result of vascular injury. The rapid onset of severe pathology in the trigeminal cells following irradiation indicates a direct relationship. This does not exclude the possibility, however, that injury of the cell is resultant upon injury of its axon rather than of the cell body itself.

Conclusions. Exposure of trigeminal ganglion cells to roentgen rays has been found to produce injury which may be separable into 3 phases:

1. Rapid phase of acute chromatolysis immediately following irradiation.
2. Recovery period.
3. Phase of the widely variable, slowly progressing spectrum of chronic chromatolysis.

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[3] Ma, W. C., and Chien-Liang Hsü, *Am. J. Cancer*, 1940, **40**, 335.

15321

Bone Growth in Paralyzed Limbs.*

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Paralysis of an extremity beginning at an early age frequently results in disturbances of bone growth. A bone of the affected extremity may be shorter than that of the normal limb and, as demonstrated by the roentgenogram, its diameter and the thickness of its cortex and trabeculae may be reduced. Pottorf[1] and Howell,[2] each of whom used one young dog as an experimental subject, investigated the changes occurring in the dimensions and structure of the bones of the forelimb after section of unspecified nerves of the brachial plexus. Howell's animal was allowed to live 19 weeks after paralysis of the right forelimb was produced and he found that the humerus and ulna of that limb were approximately 5% shorter than those of the normal limb. However, the diameter of the humerus of the paralyzed limb was much less than that of the normal limb and ap-

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* This work was supported by a grant from the Josiah Macy, Jr., Foundation.

[1] Pottorf, J. L., *Anat. Rec.*, 1916, **10**, 234.

[2] Howell, J. A., *Anat. Rec.*, 1917, **13**, 233.

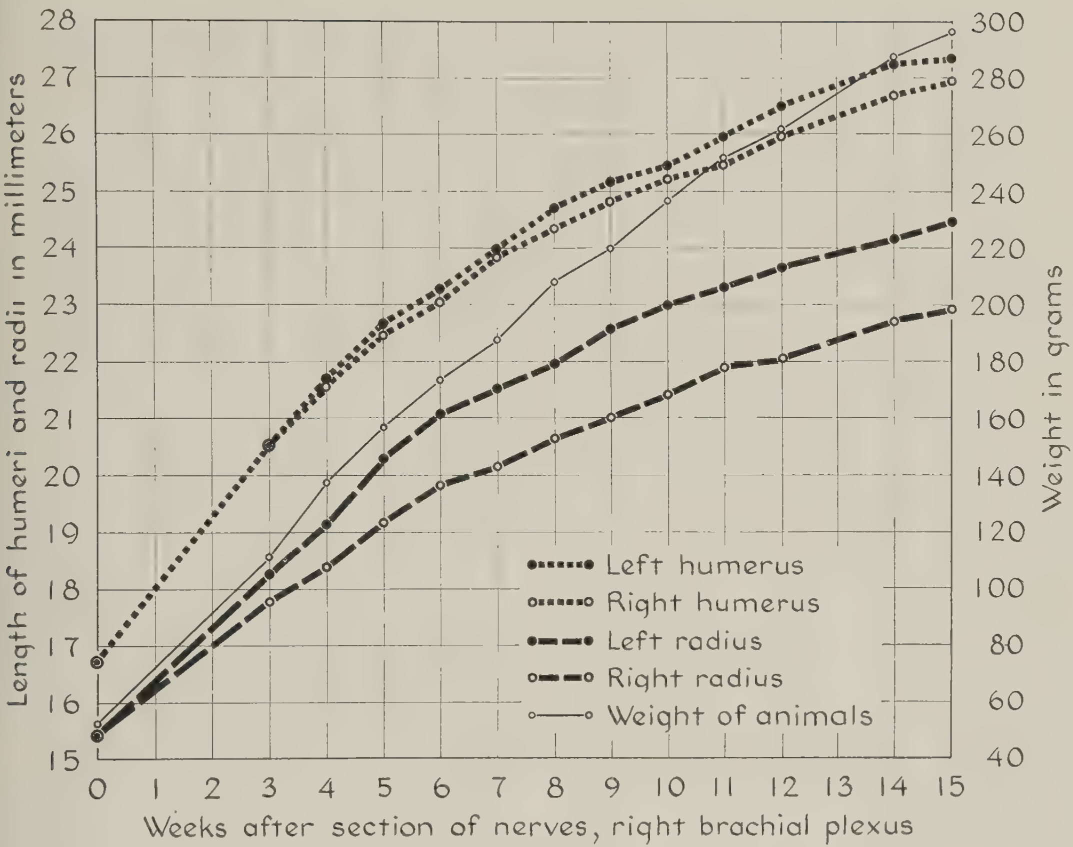

Fig. 1.
Bone growth in normal and paralyzed limbs of the rat.

peared to have suffered an actual decrease in diameter. Howell interpreted his observations to indicate that mechanical factors of stress and strain have a pronounced effect on the growth of bone, but are not the sole factors in the regulation of bone growth.

Riley, McCleary and Johnson[3] found the uptake of radioactive phosphorus by the bone ash of the humeri of normal and paralyzed limbs of rats, weighing between 150-200 g, to be equal and produced evidence to indicate that the humeri of the paralyzed limbs continued to gain in mass but at a rate slower than the normal. Allison and Brooks,[4] using roentgenographic and histologic technics, noted the gross and minute changes in the atrophic bones of the forelimbs of dogs in which paralysis was produced by section of nerves after the animals were mature. Armstrong, Knowlton and Gouze[5] determined the degree of atrophy which occurred in the humeri of mature rats following 21 days of brachial plexus paralysis and investigated the effect of certain steroid hormones on the composition and amount of bone in the humeri of the normal and paralyzed limbs.

In the present study, the length of the bones of the forelimbs was determined at intervals of time in rats in which paralysis of one arm was produced at an early age and the animals allowed to live to maturity. The initial and final diameters of the humeri were measured and data were collected to demonstrate the differences in amount of bone and of bone ash in the skeleton of the normal limbs.

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[3] Riley, F. R., McCleary, B., and Johnson, R. E., *Am. J. Physiol.*, 1945, **143**, 677.

[4] Allison, H., and Brooks, B., *Surg., Gyn., and Obst.*, 1921, **33**, 250.

[5] Armstrong, W. D., Knowlton, J., and Gouze, M., *Endocrinology*, 1945, **36**, 313.

Experimental. Eight male rats of the Sprague-Dawley strain, when 27 days of age and with an average weight of 53.9 ± 0.91 g, were subjected to an operation in which certain nerves were severed in order to produce paralysis of the right forelimb. The animals were anesthetized by the intraperitoneal injection of 50 mg sodium pentobarbital per kilo of body weight and, using aseptic technic, the following nerves of the right brachial plexus were severed at the axillary level: median, ulnar, radial, axillary, musculocutaneous, subscapular, thoracodorsal and suprascapular.[6] The animals were fed *ad libitum* for 15 weeks on a diet of 60% sucrose, 16.9% hydrogenated vegetable fat (Crisco), 18% lactalbumin (Borden's No. 15-42), 4% salt mixture (Wesson's), 1% liver extract (Wilson's Fraction D) and 0.1% choline hydrochloride. In addition, certain crystalline compounds of the vitamin B group were incorporated in the diet[†] and the animals were given twice weekly 2 drops of a concentrate of vitamins A and D. The incisor teeth were kept cut to about half normal length to prevent the animals from chewing the paralyzed extremity.

At intervals of time, beginning with the day of operation, as indicated in Fig. 1, the animals were anesthetized by the intraperitoneal injection of sodium pentobarbital and roentgenograms of both forelimbs were prepared. The extended limb, with the animal lying on its side, was held flat against the cardboard film holder and a target-to-film distance of 65 cm was used. The greatest length of the shadows of the humeri and of the radii were measured on the developed films, using a pair of finely pointed dividers and an accurately engraved ruler.[‡] These measurements were recorded to the nearest 0.25 mm. The diameters of the shadows of the humeri at the midpoint of the shaft (in the plane of the deltoid tubercle) and of the proximal epiphysis (head) of the same bones were measured on the initial and final films. These latter measurements were made with a vernier caliper which was read to the nearest 0.1 mm. All measurements of length or diameter were made by 2 observers working independently. When the results obtained by the 2 observers pertaining to a particular dimension were not identical, the measurements were repeated until agreement was reached.

The animals were sacrificed 15 weeks after the operation. The forelimbs were removed and the bones of the arms and forearms were cleaned of soft tissue after prolonged boiling in distilled water. The distal epiphyses of the radii and ulnae, which were small, were ununited to the shaft and were lost in cleaning the bones. The bones were dried and extracted for 48 hours with an equal part mixture of alcohol and ether in a Soxhlet apparatus. After the bones were redried and weighed, they were ashed in platinum dishes at 700° to constant weight.

Results and Discussion. The mean weight of the animals when sacrificed was 296.1 ± 10.2 g. Fig. 1 summarizes the results as to the rate of increase of body weight and of growth in length of the humeri and radii. The data referring to dimensions of the bones given in Table I are the mean results of measurements made from the final roentgenograms. As seen from Fig. 1, the growth in length of the radii of the paralyzed limbs was affected earlier and to a greater degree than was the case with the humeri of the

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[6] Greene, E. C., *Trans. Am. Phiol. Soc., N. S.*, 1935, **27**, 1.

[†] Per 100 g of diet: 200 μg thiamine hydrochloride, 200 μg riboflavin, 200 μg pyridoxine hydrochloride, 400 μg calcium pantothenate.

[‡] Comparison of the directly measured length of the humeri of the normal limbs with the length of the shadows of these bones on the roentgenograms made on the day the animals were sacrificed showed that the shadows were, on an average, 1.2% longer than the true length of the bones. This exaggeration of the length of bone due to divergence of the X-ray beam probably represents the largest error, due to this factor, among the measurements made from the X-ray films. The effect of divergence of the X-ray beam on the length of the shadows of the bones would be reduced in the case of the films made when the bones were shorter.

TABLE I.
Effects of Paralysis on Bones of the Forelimb of Rats.*

	Humeri		Radii		Ulnae	
	Normal limbs	Paralyzed limbs	Normal limbs	Paralyzed limbs	Normal limbs	Paralyzed limbs
Length (mm)	27.37 ±0.231	26.91 ±0.249	24.47 ±0.148	22.84 ±0.190		
Diameter of shaft (mm)	2.85† ±0.039	2.52† ±0.005				
Diameter of head (mm)	5.59‡ ±0.055	5.39‡ ±0.057				
Ash content (%)	68.69 ±0.158	68.33 ±0.147	70.63 ±0.188	69.28 ±0.343	70.80 ±0.244	69.94 ±0.287
Dry, fat-free weight (mg)	215.2 ±3.09	165.9 ±4.71	48.8 ±1.24	42.8 ±1.31	94.1 ±1.77	65.0 ±1.99
Difference (%)	22.9		27.2		30.9	
Ash weight (mg)	147.8 ±2.40	113.4 ±3.30	41.5 ±0.64	29.7 ±0.77	66.6 ±1.05	45.5 ±1.37
Difference (%)	23.3		28.4		31.7	

* The data are reported as means ± the standard error.
† Initial diameter of shaft 1.91 ± 0.037 mm.
‡ Initial diameter of head 4.32 ± 0.042 mm.

same limbs. This finding may be due to the possibility that a more complete denervation of the muscles moving the forearm was obtained than of those having an action directly, or indirectly through the scapula, on the upper arm. In this connection, it is to be noted in Table I that the forearm bones of the paralyzed limbs exhibited a greater difference of dry, fat-free weight and of ash weight from the corresponding normal bones than was the case with the humeri.

It will be seen that while the mean final length of the humeri of the paralyzed limbs was slightly (1.7%) less than that of the normal limbs, the diameters of the shaft§ and head of these bones were very much less than those of the normal humeri. Although appositional growth was reduced more than epiphyseal growth, the first process was not prevented as is seen by a comparison of the initial and final diameters of the shaft and head of the humeri. The consistently lower ash content of the bones of the paralyzed limbs is in agreement with the results of experiments in which the paralysis of one arm was produced in mature animals.[5]

It will be noted in Fig. 1 that the rate of growth of the humeri of both limbs was very greatly reduced at the end of the experiment when the animals were 132 days old. This finding indicates that these bones had reached, or very nearly reached, their maximum length, a conclusion which is strengthened by the fact that the final roentgenograms showed that the heads of the humeri had practically complete bony union with the shafts. The curves showing the rate of growth of the radii, however, indicate that the bones were still growing at the end of the experiment. As mentioned above, the distal epiphyses of the radii and ulnae were found to lack bony union to the shaft when the animals were sacrificed. These findings indicate that skeletal growth in the rat has not ceased in animals with a body weight which is usually considered to be that of a mature animal.

Summary: Fifteen weeks after the forelimbs of young rats were denervated, it was found: (1) The growth in length of the radii was affected earlier and to a greater degree than that of the humerus. (2) The reduction of growth in diameter of the humeri was greater than the reduction of growth in length. (3) Growth in diameter of the bones was reduced but not prevented. (4) The quantity of bone and of bone salt was reduced by about 25%. (5) The humeri, but not the radii, of both the normal and paralyzed limbs of rats 132 days of age, had practically ceased to grow in length.

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§ The directly determined diameters of the midpoint of the humeri measured in a plane perpendicular to that of the deltoid tubercle and at a right angle to the dimension measured from the films were: Humeri of normal limbs, 2.20 ± 0.038 mm and humeri of paralyzed limbs, 1.75 ± 0.071 mm.

It is concluded that paralysis of a limb interferes more with appositional growth of bones than with epiphyseal growth and that bone growth is not prevented in a denervated limb.

15322 P

Serial Transmission of the Milk Agent of Mouse Mammary Carcinoma*

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In a colony of inbred mice bearing breast tumors, the milk agent,[1] or virus,[2] is transmitted from mother to daughter by nursing. Mammary tumors indicating the presence of the milk agent do not appear in the mother until after the milk agent has been transmitted to the young. Thus, the milk agent in perpetuation is passed serially by nursing during the precancerous stage.

We have attempted, by artificial injection, to pass the milk agent of mouse mammary carcinoma through a series of susceptible mice in a manner similar to the usual mode of transmission in the precancerous stage. There was a decline of the tumor formation in the second passage and entire disappearance of tumors in the third passage. The mice inoculated in series were of the ZBC strain, a hybrid susceptible to the milk agent but not infected with it.

The serial inoculation was begun with a filtrate of spontaneous tumor from C3H mice. For the serial transmission of the agent, or virus, through the ZBC mice, filtrates of lactating mammary tissue were used as the inoculant. The general procedure was to inoculate 30 mice in each generation, 15 of which we then held indefinitely and force-bred in order to induce maximum production of cancer. After allowing the other 15 inoculated mice to raise one litter, we killed them to obtain lactating mammary tissue to be used for inoculation of the next 30 mice in series. Females of the litters so produced were also force-bred for observations on cancer production.

In preparing the inoculant for the original group of 30 ZBC mice, 15 g of spontaneous tumor from C3H mice was ground with a mortar and pestle and suspended in 25 cc of 0.9% saline solution. The suspension was centrifuged 10 minutes at 2000 r.p.m. so that the coarsely ground residue was removed. The supernatant fluid was then filtered through a tested Berkefeld filter, and the filtrate was injected into ZBC mice one month of age. Each mouse received by intraperitoneal inoculation a dose of 0.5 cc of filtrate, or the equivalent of 0.5 g of cancer tissue. For all subsequent inoculations, lactating glands obtained from female mice after birth of their first litter, were ground, centrifuged, filtered and injected in similar manner. The dosage, however, varied somewhat in each generation. In the first transfer generation in ZBC mice, the dose of filtrate for each animal was the equivalent of 0.25 g lactating mammary tissue. In the second generation, the dose of filtrate was equivalent to 0.33 g of mammary tissue and in the third and subsequent generations, equivalent to

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* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Cancer Research Fund of the Graduate School of the University of Minnesota, and the Citizens Aid Society of Minneapolis.

[1] Bittner, J. J., *Science*, 1936, **84**, 162.

[2] Green, R. G., Moosey, M. M., and Bittner, J. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 115.

TABLE I.
Serial Inoculations in Mice in Precancerous Period. C3H Tumor to ZBC Mice Without Milk Factor.

Serial passage	Interval between inoculations (days)	Mice injected	Mice held	No. of tumors	Time of appearance of last tumor (mo.)	Progeny produced	No. of tumors	Time of appearance of last tumor (mo.)
Original	—	30	15	15	10	34	29	8.2
1st	72	30	15	11	7.5	30	29	8.0
2nd	53	30	15	6	7.5	24	0	—*
3rd	90	30	15	0	—*	26	0	—*
4th	71	30	15	0	—*	19	0	—*

* Period of observation 7 to 12 months.

0.25 g. The appearance of tumors in the inoculated mice and in the first-litter progeny of those used for transmission material is shown with other pertinent data in Table I.

In the first group of mice all 15 females, which received the original mammary tumor filtrate, developed tumors; 29 of the 34 progeny that did not receive the injection also developed tumors. The appearance of tumors in these 2 groups in 10 months and 8.2 months, respectively, demonstrates the high susceptibility of the hybrid ZBC mice to the virus as isolated from the tumor. The number of tumors in the inoculated female mice dropped to 11 after the first transfer from lactating mammary glands, and to 6 in the second transfer, with no tumors appearing in later generations. It seems of some significance that although few tumors developed in the first and second generations, the time of their appearance was considerably shorter than in the original group and shorter than might be expected if the tumors had been spontaneous ones appearing in mice that nursed naturally infected females. This fact, together with the appearance of early developing tumors and then a sudden disappearance of tumor formation in the progeny of the second generation, makes it seem that a relationship may exist between the earlier development and the decline in the number of tumors.

The procedure followed in these experiments seems to differ from natural transmission principally in the manner of introduction of the milk agent, in the delay of a few weeks in transfer, and possibly in the dosage, both as to size and repetition. It is not clear at this time whether the disappearance of tumor formation is related to the fact that a hybrid strain of mice was used, to a loss or a modification of the virus itself, or to a change in immunologic relationships.

Summary. In serial passage of the milk agent, or virus, through highly susceptible ZBC hybrid mice by the intraperitoneal injection of mammary gland filtrates, the number of tumors declined in the first and second passages, and no tumors developed in the third and fourth passages.

15323

Metabolism of Nicotinamide, Nicotinic Acid and Diethylamide of Nicotinic Acid (Coramine) by Newborn and Premature Infants.

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Najjar and Wood[1] reported the discovery of a compound in normal urine which is rendered fluorescent by the addition of alkali and butanol. Subsequent investigators[2,3] have indicated that this substance is nicotinamide methochloride (N^1 methyl nicotinamide) and have demonstrated that its excretion is augmented by the ingestion of nicotinamide, nicotinic acid and coramine. Ellinger and Coulson[4] studied the relative rate of elimination of nicotinamide methochloride following administration of these compounds to normal adults and concluded that nicotinamide is directly methylated whereas coramine and nicotinic acid are first converted to nicotinamide and are then methylated.

Since practically nothing is known relative to the metabolism of nicotinic acid and its derivatives by newborn and premature infants the present study was undertaken. The living conditions, diet, etc., of the young patients are well standardized and consequently this study was conducted under experimental conditions which were distinctly more uniform than those which prevailed in the investigations which have been made on adults.

Experimental. Studies on Normal Newborn Infants. Colored newborn male infants were selected for the first experiment. These babies were considered full term on the basis of their general appearance and their birth weights, which averaged 7.1 lb. (5.2-9.8 lb.) The infants were maintained under the ordinary conditions which prevail in a hospital ward where aseptic nursing is practiced. The babies received sterile water the first day of life and from 4 to 8 ounces of a sterile formula consisting of ⅓ evaporated milk, ⅔ water and 5% karo corn syrup the second day. Subsequently the food intake gradually increased to a maximum of 24 ounces of the formula per 24 hours by the eighth day. Twenty-four-hour urine specimens were obtained* by fastening a rubber tube over the penis with adhesive tape and collecting the urine in a loosely stoppered bottle which contained 5 ml of glacial acetic acid as a preservative. At the close of each 24-hour period the lower abdomen was depressed manually to force as much of the residual urine as possible from the bladder. Nicotinamide, nicotinic acid or coramine was given by mouth at 8 a.m. to some of the infants whereas other babies who received no medication served as controls. The urinary methochloride was determined by the method of Coulson, *et al.*[5] which involves the adsorption of the methochloride on Decalso; elution with KCl and the subsequent development of fluorescence by alkaline isobutanol.

After experimenting with a number of nicotinamide "saturation" tests in adults Ellinger and Benesch[6] adopted the total 24-hour output of nicotinamide methochloride following a single oral test dose of 100 mg of nicotinamide as perhaps the most reliable index of the nutritional level with respect to

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[1] Najjar, V. A., and Wood, R. W., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 386.

[2] Huff, J. W., and Perlzweig, W. A., *Science,* 1943, **97**, 538.

[3] Ellinger, P., and Coulson, R. A., *Nature,* 1943, **152**, 383.

[4] Ellinger, P., and Coulson, R. A., *Biochem. J.,* 1944, **38**, 265.

* We are indebted to Miss Hazel Pierce and Miss Tallulah Moore for aid in the collection of urine samples.

[5] Coulson, R. A., Ellinger, P., and Holden, M., *Biochem. J.,* 1944, **38**, 150.

[6] Ellinger, P., and Benesch, R., *Lancet,* 1945, **2**, 197.

TABLE I.

Nicotinamide Methochloride Output in mg/24 Hours Without Medication or Following Oral Administration of Nicotinamide, Nicotinic Acid or Coramine.

	None			100 mg			100 mg		None			
Medication given	No. of cases	Avg age hr	Avg daily output mg	No. of cases	Avg age hr	Output 1st day mg	No. of cases	Output 2nd day mg	No. of cases	Output 3rd day mg	No. of cases	Total for 3 days mg
A. Full-term newborn infants												
None	23	13	3.2 (0.3-7.4)									
N. Amide				15	16	31.2 (12.1-72.1)	15	51.7 (10.3-93.0)	15	25.8 (2.8-54.7)	15	108.7 (28.9-178.5)
N. Acid				11	14	14.7 (3.0-28.5)	11	29.8 (2.5-57.5)	11	20.8 (7.5-42.0)	11	65.3 (20.0-120.8)
Coramine*				11	20	4.9 (1.3-8.8)	11	6.8 (1.3-9.6)	11	12.0 (5.2-22.7)	11	23.7 (7.8-37.2)
B. Premature infants												
	None			100 mg			100 mg		None			
		days			days							
N. Amide	7†	11	1.7 (0.0-4.4)	7	12	20.5 (1.5-87.0)	7	37.8 (3.8-133.0)	7	13.5 (2.6-25.0)	7	71.9 (10.6-236.6)
N. Amide	15‡	11	1.7 (0.0-5.6)	13	12	22.5 (1.5-87.0)	11	36.5 (3.8-133.0)				

* Coramine was administered in doses equivalent to 100 mg nicotinamide on the basis of molecular weight.

† This series represents the data from 7 prematures from whom we were able to obtain 24-hour urine samples for 4 consecutive days.

‡ This series includes the 7 above cases and several additional cases from whom we were unable to obtain 4 consecutive 24-hour urine samples.

this item. The present authors applied this test to 24 full term newborn infants for the first or preliminary experiment. These infants whose records do not appear in the tables eliminated an average of 36.7 mg (11.5-95.0 mg) of the methochloride in the 24-hour period which immediately followed the oral administration of the single test dose. This excretion was more than 10 times that eliminated per 24 hours by control infants (Table I A) who did not receive nicotinamide and who served as normal controls for later studies.

It was our opinion that the relative degree of "saturation" might better be determined by analysis of the 24-hour urine samples following 2 successive doses given 24 hours apart. Consequently additional experiments were conducted on infants who received either 100 mg of nicotinamide or nicotinic acid or doses of coramine which on the basis of its molecular weight were equivalent to 100 mg of nicotinamide. These compounds were administered on each of the first 2 days of life. On the following day, during which no medication was administered, a third urine sample was collected and its methochloride content was also determined. The daily urinary titers of nicotinamide methochloride excreted by these cases as well as by the controls who received no medication appear in Table I A.

The total nicotinamide content of the small quantity of formula the infants consumed probably was negligible if we are to accept the values Cheldelin and Williams[7] obtained for the nicotinamide content of cow's milk. Apparently the intestinal flora or the tissue stores were the chief sources of nicotinamide for these infants until it was administered orally.

The 23 control infants eliminated an average of 3.2 mg of the methochloride during the first day of life (Table I A). This average is distinctly lower than those observed for the first 24-hour period following the administration of nicotinamide, nicotinic acid or coramine. The data in Table I A indicate that nicotinamide has a greater immediate influence on elevating the methochloride output than nicotinic acid and that nicotinic acid is considerably more effective in this respect than coramine. These observations for infants are in essential agreement with the findings of Coulson and Ellinger[4] as to the relative influence of these compounds on the elimination of the methochloride in adults.

For each of the experimental groups the output of nicotinamide methochloride was significantly higher on the second day of medication than on the first day. On the third day during which no medication was given the urinary titer decreased considerably for the full term infants who had received nicotinamide or nicotinic acid but increased significantly for the group to which coramine was administered. Our results also show that with respect to their effect on the elimination of nicotinamide methochloride the relative positions of these 3 compounds remained unchanged throughout the 3-day period of observation covered by this study.

It would seem that like the adult, the newborn infants are able to methylate the preformed nicotinamide with relative ease and rapidly whereas they convert coramine slowly and somewhat less completely first to nicotinamide and then to nicotinamide methochloride. Nicotinic acid occupies an intermediate position in this regard. The absolute range of values for the methochloride elimination was very great in the nicotinamide and nicotinic acid groups but varied within more narrow limits in the infants who received coramine.

Premature Infants. Although special difficulties attend experimental studies on premature infants, nevertheless we obtained some information on their methochloride elimination before and following the administration of nicotinamide. In each instance an attempt was made to collect 4 consecutive 24-hour urine specimens, the first without the administration of nicotinamide, the second and third following 2 daily doses of 100 mg each and the fourth without medication. Complete observations were made in 7 cases (Table I B) but owing to vomiting and other disorders common to premature infants the data from the remaining 8 infants included in the study are incomplete. On the first day of the experiment the average age and weight of these infants was 11 days and 4.2 (3.2-5.0) lb., respectively. The quantity of food these infants received varied according to age and body weight, but in only one instance did the cow's milk consumption exceed 250 cc in 24 hours. It is apparent that the diet furnished them with less than a milligram of nicotinamide a day.

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[7] Chedelin, V. H., and Williams, R. R., *Jour. Nutrition*, 1943, **26**, 417.

The results of the estimation of the methochloride elimination by the premature infants appear in Table I B. The average value for the first day of the study (11th day of life) was distinctly lower than that for the 23 newborn full term control infants. Throughout the period of study the increase in the excretion of nicotinamide methochloride following the administration of nicotinamide to the premature infants also averaged consistently below that observed in the full term infants given equal doses of the compound. Although one premature eliminated 236.6 mg of the methochloride after a total intake of 200 mg of nicotinamide this was the only case where the excretion over the 3-day period exceeded 71.9 mg, the average total excretion for the entire group (Table I B). In spite of the fact that as compared with premature infants the absolute excretion was much greater in the case of the normal newborn infants the relative per cent of the 3-day total excreted each day was nearly the same in both normal infants and prematures. No correlation was found between the total methochloride elimination and the daily urine volumes. The average urine volume of the prematures was 85.4 ml/24 hours and that of the newborn full term infants 91.6 ml on the days of the experiment.

Effect of Methionine on Nicotinamide Methochloride Elimination. The role of methionine in methylation has been studied by Perlzweig *et al.*[8] who have presented evidence that this substance is involved in the methylation of nicotinamide. We recognized the possibility that the low values for nicotinamide methochloride excretion obtained for some of our infants might be due to a deficiency in their body store of methionine rather than in the inherited store of nicotinamide. Consequently 8 infants were given 0.3 g each of methionine 20 hours before the experiment started and an additional 0.3 g at the time the 100 mg dose of nicotinamide was administered. The results which appear in Table II show an average 24-hour excretion of 32.4 mg of nicotinamide methochloride. Since this value does not differ significantly from that observed for the 15 infants who received 100 mg of nicotinamide but no methionine (Table I A) it appears that in the quantity given methionine had no appreciable effect on the excretion of nicotinamide methochloride. It is possible that larger doses of methionine administered over a longer period might yield different results.

TABLE II.
The 24-Hour Nicotinamide Methochloride Output of Newborn Infants Following One Dose of 100 mg of Nicotinamide and 0.6 g Methionine.

Case	24 hr urine vol.	Nicotinamide methochloride output in mg/24 hr
M. B.	125	37.5
E. R.	205	28.7
O. S.	84	65.5
M. T.	118	48.4
L. D.	156	27.3
L. T.	87	15.2
M. C.	90	24.8
H. F.	38	11.4
	Avg 112.9 ml	Avg 32.4 mg

The Metabolism of Coramine. The slow and relatively incomplete conversion of coramine to what is apparently nicotinamide methochloride leads us to suspect that coramine might be eliminated, at least in part, as coramine methochloride. To test this hypothesis coramine methochloride was synthesized by the methylation of coramine in methanol by methyl iodide, and the subsequent conversion of the iodide to the chloride by the action of silver chloride. Coramine methochloride proved to be non-fluorescent in the filtered 366 mu line from a quartz mercury vapor lamp. The addition of NaOH and isobutanol converts coramine methochloride into a fluorescent derivative, the hue of which is indistinguishable from that developed from nicotinamide methochloride by the same procedure. Coramine methochloride also resembles nicotinamide methochloride in that it is adsorbed on Decalso, eluted with KCl and as a consequence it may be estimated fluorometrically in the same manner as nicotinamide methochloride. Preliminary experiments have shown that the absolute intensity of the fluorescence of the derivative of coramine is, however, only about 12% of that developed from equivalent amounts of nicotinamide methochloride.

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[8] Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, 1943, **150**, 401.

In order to test the effect of coramine methochloride on the fluorescence developed from the urine by the procedure of Coulson *et al.*[5] for the estimation of nicotinamide methochloride an adult was given a single oral dose of 200 mg of coramine methochloride. No increase in fluorescence occurred. As a further check 2 normal newborn infants were each given 200 mg of coramine methochloride subcutaneously without increasing the total fluorescence developed from the urine by alkaline-isobutanol treatment. It would appear that if coramine is methylated in the human body and excreted as coramine methochloride this does not occur to any appreciable extent. It is more probable that coramine is normally converted to nicotinamide which in turn is methylated and excreted.

Discussion. The nicotinamide produced by the intestinal flora is an important if not the chief source of this vitamin for the adult.[9,10] Since, however, the average age of our newborn infants was less than 40 hours at the conclusion of the first day of the experiment, it is doubtful that the intestinal flora was sufficiently well established to provide them with any appreciable quantity of nicotinamide. Furthermore, since they received nothing but water for 24 hours and very small quantities of milk during the succeeding 10 hours they derived very little of the vitamin from the diet. Nevertheless, in spite of scant nicotinamide production in the gut and a very low dietary intake the newborn infants studied excreted 3.2 mg nicotinamide methochloride in the urine, a quantity probably in excess of that derived from these 2 sources.

The nicotinamide which appears in the urine as the methochloride represents nicotinamide which was either synthesized by the infant or which diffused across the placenta to the fetus before parturition. The great variations in the daily methochloride excretions of newborn infants probably reflect either variations in the nutritional status of the mother or different degrees of permeability of the placental barrier. It is premature to suggest that those infants whose methochloride elimination was distinctly below the average found in this study are necessarily "deficient" in the pellagra-preventive compounds since very little is known of the absolute human adult nicotinamide requirements and nothing is known of the newborn. Since the methochloride content of newborn infants probably exceeds that which could have been attributed to nicotinamide derived from the diet or intestinal flora it is probable that the tissues of the newborn infant contain an appreciable store of nicotinamide at the time of birth.

The excretion of nicotinamide methochloride by the full term infants following the ingestion of nicotinamide, nicotinic acid or coramine is essentially equal to that observed by Ellinger and Coulson[4] for adults when given equal doses of the same compounds. This suggests that the absolute weight of normal liver tissue is not a factor in methylation. We can offer no explanation for the observation that under similar experimental conditions premature infants excrete less nicotinamide methochloride than full term infants.

Summary. Experiments were conducted on newborn infants to determine the 24-hour elimination of nicotinamide methochloride under normal conditions or following the administration of nicotinamide, nicotinic acid or coramine. With regard to the influence of the 3 compounds tested on nicotinamide methochloride output nicotinamide proved to

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[9] Ellinger, P., Coulson, R. A., and Benesch, R., *Nature*, 1944, **154**, 270.

[10] Ellinger, P., Benesch, R., and Kay, W. W., *Lancet*, 1945, **1**, 432.

be the most active followed by nicotinic acid and then coramine. Premature infants eliminated less nicotinamide methochloride than normal newborn infants under the same conditions. Evidence is presented which indicates that coramine is normally converted to nicotinamide and excreted as nicotinamide methochloride. Under the conditions of the experiment methionine had little or no effect on the methylation of nicotinamide.

15324

Vitamin E Levels in Maternal and Fetal Blood Plasma.

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Some insight into the role of vitamins in human physiology may be gained from a study of the concentration of vitamins in body tissues and fluids in various physiologic states. Among these are pregnancy, parturition and the neonatal state. The content of vitamin E in these states is of particular interest because of its well established function in insuring normal reproduction in certain laboratory animals.

Vitamin E is normally not present in the urine.[1] Plasma, however, is readily available for investigation of vitamin E content.

The plasma levels of vitamin A in pregnant women and newborn infants have been reported in several papers.[2] These determinations became possible with the development of suitable clinical methods for determination of plasma vitamin A. A clinical method for the determination of plasma vitamin E[3] has made possible a similar investigation of the maternal and fetal plasma levels.

The plasma levels of vitamin C, carotene, vitamin A and vitamin E have been simultaneously determined in a fairly large series of normal persons and patients suffering from various disease processes. The levels found for vitamin A, carotene and vitamin C were in good general agreement with those previously reported. This report deals only with the levels found for vitamin E in the plasma of pregnant and parturient women and newborn infants.

Procedure and Methods. Plasma was obtained from oxalated blood from the cubital veins of women and from oxalated blood from the umbilical cord for infants' samples. The umbilical cord was clamped and divided a few minutes after delivery and the blood collected from the placental end of the cord before delivery of the placenta. The sample from the mother was obtained immediately after completion of the delivery—about one-half hour later than the cord sample. Vitamin E levels were determined by the method of Quaife and Harris,[3] using the hydrogenation technic of Quaife and Biehler.[4] A Coleman universal spectrophotometer model 11 was used. In brief, the method comprises extraction of plasma lipids with ethanol and petroleum ether, hydrogenation to obviate interference due to carotenoids and vitamin A, and application of the Emmerie and Engel color reaction to measure total tocopherols

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[1] Hines, L. R., and Mattill, H. A., *J. Biol. Chem.*, 1943, **149**, 549.

[2] Abt, Arthur F., and others, *Quart. Bull. Northwestern U. Med. School*, 1942, **16**, 245; Lund, C. J., and Kimble, M. S., *Am. J. Obstet. and Gynec.*, 1943, **46**, 207; Byrn, Jane N., and Eastman, N. J., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 132; Bodansky, Oscar, Lewis, J. M., and Lillienfeld, M. C. C., *J. Clin. Invest.*, 1943, **22**, 643.

[3] Quaife, M. L., and Harris, P. L., *J. Biol. Chem.*, 1944, **156**, 499.

[4] Quaife, M. L., and Biehler, R., *J. Biol. Chem.*, 1945, **159**, 663.

(vitamin E). Pure natural α-tocopherol was used to prepare a standard curve, since β- and γ-tocopherols give substantially the same color response under the conditions used.

Results with Statistical Analysis. Values in Pregnant Women. Plasma vitamin E values were determined on 23 women in various stages of pregnancy. Inspection of the results showed an increased level of tocopherols in the latter part of pregnancy. Eleven women in the first 24 weeks of pregnancy had a mean level of 1.17 mg% tocopherols with a standard deviation of 0.19. Twelve patients in the 25th to 36th week of pregnancy had a significantly higher mean level of 1.62 mg% with a standard deviation of 0.31. For men and non-pregnant women mean levels of plasma vitamin E of 1.05 mg% for a group in Rochester, New York, and 1.04 mg% for a group in Birmingham, Alabama, have been determined by the same method.[5]

Values in Mothers and the Newborn. Plasma vitamin E was determined on 54 mothers and respective cord bloods at delivery. Inclusion of 2 sets of twins gave 56 values for infants. The mean maternal vitamin E was 1.70 with a standard deviation of 0.30. The mean fetal vitamin E was 0.34 with a standard deviation of 0.12.

Thus, vitamin E is similar to other fat-soluble vitamins, A and carotene, in showing markedly lower values in the infant than in the mother. The mean ratio of plasma vitamin E in the mother to that in the infant was 5.7. This figure is interesting in view of the conclusion of Mason and Bryan,[6] based on bioassay of tissues, that for the rat "the concentration of vitamin E in the placenta and uterus of high-E mothers was about 5 times greater than in the full-term fetus or newborn."

The correlation coefficient of maternal and fetal vitamin E levels was calculated and showed absolutely no correlation between the 2.

Relationships of Fetal Vitamin E. The possibility of a sex difference in the plasma vitamin E levels of the newborn was investigated. Thirty female infants had a mean of 0.355 (S. D. = 0.145), and 26 males a mean of 0.318 (S. D. = 0.089). However, the difference is not statistically significant.

A lack of correlation between vitamin E in the infant and birth-weight was also observed.

Two sets of twins are included in the group. One pair, identical girls, showed vitamin E values of 0.42 and 0.48. A second pair, boy and girl, had identical levels of 0.30.

Comment. These results differ considerably from the levels reported by Varangot.[7] Varangot found that although the level in non-pregnant women was considerably lower than that in pregnant women, the level in the first trimester of pregnancy was higher than in the third. In 18 samples of blood collected from the umbilical vein at birth the vitamin E level was approximately 0.1 mg%. In 6 samples the amount was too small to measure. The serum vitamin E levels for non-pregnant women first reported by Varangot were much higher than those obtained with a later method in which he employed a chromatographic adsorption column to eliminate interfering substances. This method was used for the series referred to above. His results, however, agree with ours in respect to the much lower concentration of vitamin E in the fetal blood than in the maternal blood and the much higher concentration of vitamin E in the blood of pregnant than of non-pregnant women.

It has been shown that the blood lipids increase in pregnancy.[8] Vitamin E accordingly behaves in a similar way. The concentration of lipids in the fetal plasma is generally much lower than in the maternal plasma[9] which is likewise true of vitamin E.

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[5] Harris, P. L., Jensen, J. L., Hickman, K. C. D., and Spies, T., *Am. J. Pub. Health*, 1946, **36**, 155.

[6] Mason, K. E., and Bryan, W. L., *J. Nutrition*, 1940, **20**, 501.

[7] Varangot, J., *Compt. rend. Acad. d. sc.*, 1942, **214**, 691; Varangot, J., Chailley, H., and Rieux, N., *Compt. rend. Soc. de biol.*, 1943, **137**, 210; *Ibid., Compt. rend. Soc. de biol.*, 1943, **137**, 393.

[8] Tyler, M., and Underhill, F. P., *J. Biol. Chem.*, 1925, **66**, 1.

[9] Slemons, J. M., and Stander, H. J., *Bull. Johns Hopkins Hosp.*, 1923, **34**, 7; Boyd, Eldon M., and

This relationship has been shown to hold also for vitamin K as measured by prothrombin levels.[10]

While this placental barrier may be regarded usually as a protective mechanism, the fact that neonatal hemorrhages may be prevented by the administration of additional vitamin K to mothers, whose plasma contains adequate amounts of vitamin K for their own needs, might indicate that the placental barrier does not always function in a beneficial way.

Administration of supplements of vitamin E to the mother during pregnancy might possibly increase the fetal plasma vitamin E. Experiments to test this point are in progress.

"The umbilical vessels do offer a unique source of blood for analyses, but the results obtained therefrom are a little too limited to an especially transient state of affairs to throw much light on the whole period."[11] The situation at delivery does not represent the relationship throughout gestation and broad metabolic and nutritional generalizations should not be drawn from determination of this kind although they are of interest in their own right and their significance becomes greater with advancing knowledge.

Conclusions. 1. The plasma vitamin E level tends to rise with the progress of pregnancy and is approximately 65% higher at term than that for non-pregnant women.

2. The vitamin E level in the cord blood of infants at birth is much lower than the level in the venous blood of the mothers.

3. No sex difference in vitamin E levels is apparent in newborn infants.

Wilson, Karl M., *J. Clin. Investigation*, 1935, **14**, 7.

[10] Smith, H. P., and Warner, E. D., Vitamin K: Clinical Aspects, in Evans, E. A., Jr.: *The Biological Action of the Vitamins*, University of Chicago Press, 1942, 211.

[11] Smith, Clement A., *The Physiology of the Newborn Infant*, ed. 1, Charles C. Thomas, 1945, 5.

15325

Biosynthesis of Nicotinamide in Man.*

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In previous studies[1,2] we have observed that, under certain conditions at least, synthesis of thiamine and of riboflavin occurred in the gastrointestinal tract of man. These studies were carried out on purified diets in which the B factors were supplied almost entirely in the form of crystalline supplements. The recovery of these factors from the excreta was found to be in excess of the intake and in a number of experiments the excretion in the stools alone exceeded the quantity ingested. It was found that the administration of sulfasuxidine inhibited thiamine synthesis effectively, but caused no suppression of riboflavin synthesis; indeed, the synthesis of riboflavin appeared to be increased by the drug.

Experiments pointing to the synthesis of nicotinamide in the human intestine were

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* The expenses of this study were defrayed in part by grants from the John and Mary R. Markle Foundation, the Sugar Research Foundation and from Mead Johnson & Co.

[1] Najjar, V. A., and Holt, L. E., Jr., *J. A. M. A.*, 1943, **123**, 683.

[2] Najjar, V. A., Johns, G. A., Medairy, G. C., Fleischmann, G., and Holt, L. E. Jr., *J. A. M. A.*, 1944, **126**, 357.

reported by Ellinger and his collaborators.[3,4] These authors did not employ purified diets but attempted to maintain a constant nicotinamide intake with natural foods containing nicotinamide. Measurements were made of the excretion of N-methyl nicotinamide derivatives (F_2) in the urine, which were found to decrease markedly after the administration of sulfa drugs, notably sulfasuxidine; from this the authors deduced that a substantial amount of nicotinamide was being synthesized by the intestinal bacteria.

It seemed to us desirable to study the biosynthesis of nicotinamide under conditions in which the intake was rigidly controlled and reduced to a minimum—namely, with purified diets. It is the purpose of the present communication to report such studies. We have been able to confirm the finding of Ellinger *et al.* in regard to the biosynthesis of nicotinamide, but not in regard to the suppression of this phenomenon by sulfasuxidine.

Experimental. Subjects. Studies were made on 4 adolescents between 10 and 14 years of age who were inmates of an institution. The subjects lived a sedentary life during the period of study—approximately 3 months. Temperature and pulse were recorded 4 times daily and physical examinations were done about once a week. The subjects remained in excellent health throughout the study; they were examined with particular care for changes suggestive of pellagra, but no such changes were observed.

Diet. This consisted of vitamin-free casein,[†] crisco,[‡] dextrimaltose[§] and minerals[||] mixed together to form a dough which was fed in equal quantities at each of the 3 meals. This diet provided approximately 40 calories per kilogram, distributed as follows: protein 15%, fat 35% and carbohydrate 50%. The mineral mixture was supplied in quantities of 1.5 g daily. In order to insure accuracy of intake vitamin supplements were given independently. Fat soluble vitamins were provided by a daily dose of 5 drops of cod liver oil concentrate (Mead Johnson). A mixture of water soluble vitamins[¶] containing no nicotinic acid was given in equal quantities 3 times a day. Small amounts of nicotinic acid were found to be present in the basal diet, providing a maximum intake of 1.5 to 2.0 mg per day.

Plan of Study. The study was divided into 3 periods of approximately one month each. During the first and third periods the nicotinic acid deficient diet was given alone; during the second period sulfasuxidine was added in doses of 1.5 g every 4 hours.

Urine was collected throughout and 24-hour specimens were analyzed daily for N-methyl nicotinamide by the fluorometric method of Najjar.[5]

Results. The output of N-methyl nicotinamide (F_2) in the urine for the 4 subjects is presented in Table I where daily average figures for each 6 days of the study are shown.

It will be noted that the output of N-methyl nicotinamide on the experimental diet averaged close to 1 mg per day (extremes 0.16-2.25 mg), being somewhat less than values reported for active male adults on a

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[3] Ellinger, P., Coulson, R. A., and Benesch, R., *Nature*, 1944, **154**, 270.

[4] Ellinger, P., and Benesch, R., *Lancet*, 1945, **1**, 432.

[†] The vitamin-free casein (Sheffield) used in the study contained 0.3 to 0.9 γ nicotinic acid per g, according to assays made in the Sheffield laboratories.

[‡] This was supplied through the courtesy of Procter and Gamble.

[§] Dextrimaltose No. 2, used in this study was generously supplied by Mead Johnson and Co. Nicotinic acid assays of this product, for which we are indebted to Dr. Warren M. Cox, Jr., showed a content of 8.6 γ per g.

[||] The mineral mixture used had the following composition (measured in g): NaCl 18.9, $CaHPO_4$ (anhydrous) 25.0, $MgSO_4$ (anhydrous) 6.86, $KHCO_3$ 44.4, KCl 2.88, Fe_3Citrate 2.21, $CuSO_4$ (anhydrous) 0.24, $MnSO_4$ (anhydrous) 0.15, KI 0.015 and NaF 0.03.

[¶] The mixture of water soluble vitamins provided the following daily quantities; ascorbic acid 25 mg, calcium pantothenate 1 mg, pyridoxine 1 mg, inositol 1 mg, *p*-aminobenzoic acid 1 mg, choline chloride 5 mg, thiamine 1 mg, and riboflavin 1 mg.

[5] Najjar, V. A., *Bull. Johns Hopkins Hosp.*, 1944, **74**, 392.

TABLE I.
Urinary Output of N-Methyl Nicotinamide Chloride (μg per Day).

The figures represent average daily excretions in successive 6-day periods

Diet	Date	Subjects			
		Bu.	Ge.	Mi.	Sc.
Foreperiod on institutional diet	10-15 to 10-20-'44	960	334	†	902
Purified diet started	10-21				
Part I. *Nicotinamide-deficient diet*	10-22 to 10-27	463	270	†	655
	10-29 to 11-3	1065	246	†	1683
	11-4 to 11-9	555	240	†	767
	11-10 to 11-15	337	166	580	873
	11-16 to 11-21	223	179	265	684
Sulfasuxidine started	11-25				
Part II. *Nicotinamide-deficient diet plus sulfasuxidine*	11-28 to 12-3	233	871	675	956
	12-4 to 12-9	526	674	527	855
	12-10 to 12-15	685	790	1404	1087
	12-16 to 12-21	1914	2131	2250	1582
	12-22 to 12-26*	1852	1642	1649	1792
Sulfasuxidine stopped	12-26				
Part III. *Nicotinamide-deficient diet*	12-27 to 1-1-'45	1599	874	1965	1582
	1-2 to 1-7	649	408	1094	1105
	1-8 to 1-13	509	†	852	1481
	1-14 to 1-19	251	471	840	1362

* This period represents an average of 5 days only.
† Specimens lost during this period.

normal diet (2 to 10 mg)[6,7,8] and for control subjects on our purified diet (avg 4.28 mg) who received a daily supplement of 25 mg nicotinamide. However, the output on our experimental diet was comparable to that on the institutional diet, which was calculated to contain more than 10 times as much nicotinic acid. In other words *a 10-fold reduction in nicotinic acid intake produced no appreciable reduction in the output of the methyl derivative in the urine.*

Under conditions observed hitherto by various workers only a fraction of the ingested nicotinamide, varying from 10 to 40% appears in the urine as N-methyl derivative. In the present experiments, however, it is evident that at times the urinary excretion of N-methyl derivative is comparable to that of the ingested nicotinamide. This might be interpreted as indicating either: (1) that at the low level of intake in these experiments close to 100% of the ingested nicotinamide was excreted in the urine as the N-methyl product or (2) that a substantial synthesis of nicotinamide occurred within the body. We are inclined to dismiss the former possibility, because of the numerous observations now available that pellagrins on pellagra-producing diets and dogs on a black-tongue-producing diet show negligible quantities of N-methyl nicotinamide in the urine. Hence a reduction of nicotinamide intake *per se* is not followed by a decreased proportion of nicotinamide excretion in the form of N-methyl derivative and by the persistence of that derivative in the urine in considerable quantity, as occurred in the experiments here reported. The persistent excretion must therefore be attributed to biosynthesis of nicotinamide.

The effect of sulfasuxidine, which failed entirely to cause any reduction in N-methyl nicotinamide output, was somewhat of a surprise to us in view of the sharp reduction in output that had followed its use in the experiments of Ellinger *et al.* Indeed it ap-

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[6] Johnson, R. E., Henderson, C., Robinson, P. F., and Consolazio, F. C., *J. Nutr.*, 1945, **30**, 89.

[7] Ellinger, P., and Benesch, R., *Lancet*, 1945, **1**, 197.

[8] Mickelsen, O., and Erickson, L. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 33.

peared that in 3 of our 4 subjects there was an increased excretion of N-methyl derivative in the sulfasuxidine period with a tendency to decline when the drug was discontinued. The explanation of this discrepancy may well be in the nature of the basal diets, which permitted different bacterial flora to flourish when the coliform organisms were suppressed by the drug. In our experiments it may be assumed that the organisms which replaced the colon bacilli were capable of synthesizing nicotinamide whereas under the conditions of the British investigators organisms flourished which were incapable of performing this synthesis.

In a recent communication Mickelsen and Erickson[8] reported some human experiments in which a considerable reduction in nicotinamide intake was followed by only a minimal reduction in the output of N-methyl derivative. This led them to question the value of F_2 excretion "as a means of evaluating the state of niacin nutrition." We believe that their data deserve a different interpretation in the light of our observations and those of Ellinger on the biosynthesis of nicotinamide. Under conditions in which biosynthesis occurs there is apparently little correlation between niacin *intake* and F_2 excretion, but we see in their data nothing to disprove the view that niacin *nutrition* is not reflected in the F_2 output. As far as we are aware the subjects studied by these authors even on a minimal niacin intake failed to develop pellagra or any other manifestation attributable to niacin deficiency. The F_2 output would seem to be a better criterion of the adequacy of niacin nutrition than a niacin assay of the diet.

Summary. (1) Four subjects subsisted for a period of 3 months on a diet of purified vitamin-free foods furnishing a daily intake of 1.5-2.0 mg nicotinamide. (2) There were no symptoms or signs of nicotinic acid deficiency observed during this period. (3) The urinary output expressed as N-methylnicotinamide chloride showed no reduction suggestive of depletion of nicotinamide stores, a finding which suggests synthesis of the vitamin by the intestinal bacteria. (4) An attempt to inhibit bacterial synthesis of nicotinamide in the intestinal tract was not reflected by a drop in the urinary excretion of N-methylnicotinamide (F_2). (5) This suggests that nicotinamide synthesis in the intestinal tract was accomplished by bacteria which were not sensitive to sulfasuxidine in the doses used.

15326 P

Functional and Fatty Changes in Liver During Chronic Vitamin B Complex Deficiency.*

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A decrease in hepatic function has been reported in dogs when a diet producing blacktongue is fed[1] or during an acute deficiency of the vitamin B complex.[2] Thus, vitamin B complex may play a role in maintaining normal liver function. In such animals the decreased protein intake may also be a factor in producing hepatic dysfunction. The reduction of the protein intake of dogs to zero produces dye retention.[3]

In the present study a *chronic* deficiency of the *whole* vitamin B complex was produced. By using appropriate inanition controls the effect of the vitamin deficiency state

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*Aided by a grant from Eli Lilly and Company, Indianapolis, Indiana, and the Fluid Research Fund of Yale University School of Medicine.

[1] Rhoads, C. P., and Miller, D. K., *J. Exp. Med.*, 1938, **67**, 463.

[2] Drill, V. A., Shaffer, C. B., and Leathem, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 328.

[3] Hough, V. H., Monahan, E. P., Li, T. W., and Freeman, S., *Am. J. Physiol.*, 1943, **139**, 642.

TABLE I.
Food Intake and Liver Function of Chronic Yeast-Deficient Dogs.

Weeks	Food intake: Cal./sq M/hr	Food intake: % of normal dogs	Bromsulphalein ret'n.* %	Serum phosphatase* units
41% casein (Exp. 1)				
0-5	46-63	65-103	2-6	2.5-4.5
6-10	42-49	64-100	2-3	3.5-4.0
11-20	32-47	37-74	3-5	2.0-5.0
21-30	15-35	23-45	3-5	2.5-5.0
31-39	17-35	29-55	3-5	2.2-6.0
20% casein (Exp. 2)				
0-5	50-83	86-120	3-4	2.0-4.0
6-10	22-51	31-69	3-16 (1)	2.5-4.0
11-20	18-44	34-68	3-18 (2)	3.0-6.0
21-27	37-58	44-86	4-7 (1)	4.0-7.5 (1)
28-31	34-45	60-71	2-19 (2)	2.5-6.5 (1)
31-33	25-34	49-60	12-60 (3)	4.0-10.0 (2)
34-37	22	29-46	3-10	2.0-4.0
38-42	26	34-48	3-7	2.0-5.0

* Upper limit of normal: bromsulphalein retention, 6%; serum phosphatase, 5.0 units. Parentheses indicate number of dogs showing change from normal.

on hepatic function was determined.

Methods. A total of 16 adult dogs received a synthetic diet containing either 41% or 20% casein.† The amount eaten during a 3-hour period each day was averaged for the week and calculated as calories consumed per square meter of surface area per hour. Three chronic vitamin B complex deficient dogs in each study received 0.1 g yeast 2019 per kilo per day. Three normal control and 2 inanition control dogs, used in each study, received 4.0 g type 3 yeast extract per day,‡ which supplied a normal intake of B vitamins. Normal control dogs were used to measure normal fluctuations in food intake. Control inanition dogs were limited to the same number of calories, per square meter of surface area, consumed by the chronic B complex deficient animals the previous week. Liver function was studied by means of alkaline serum phosphatase[4] and bromsulphalein[5] (5 mg/k) determinations.

Results. Experiment 1. The chronic B complex deficient animals showed a progressive decrease in food intake which fell to 29-55% of normal (Table I). During the 39 weeks of low yeast supplement all groups of dogs maintained a normal bromsulphalein clearance and normal serum phosphatase.

Experiment 2. The chronic B complex deficient animals, receiving the same yeast intake as in Experiment 1 but with a 20% casein diet, also showed a decrease in food intake (Table I). Changes in bromsulphalein retention and serum phosphatase occurred in 2 of the 3 dogs before the 27th week (Table I). This finding is in contrast to the lack of changes in Experiment 1 during the same period, when a similar yeast intake was given but with a 41% casein diet.

On the 27th week the yeast intake of the deficient dogs was reduced by one-half. This

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† Diet consisted of: casein, 20; lard, 21.5; bone ash, 2.6; salt mixture (Karr), 1.3; and sucrose, 54.6%, plus 20 drops of oleum percomorphum per kilo of diet. When the casein was increased to 41% the sucrose was correspondingly reduced.

‡ Yeast 2019 contained per g: thiamine, 18-30 γ; riboflavin, 65 γ; pantothenic acid, 150 γ; nicotinic acid, 550 γ; pyridoxine, 40 γ; choline, 4 mg; inositol, 4 mg; and biotin, 2.5 γ. Type 3 yeast extract contained per g: thiamine, 900 γ; riboflavin, 210 γ; pantothenic acid, 450 γ; nicotinic acid, 4.0 mg; pyridoxine, 150 γ.

The authors wish to thank Mr. R. F. Light of the Fleischmann Laboratories for the analyzed yeast, and Dr. C. E. Bills of Mead Johnson and Company for the oleum percomorphum.

[4] Bodansky, A., *J. Biol. Chem.*, 1937, **120**, 167.

[5] Rosenthal, S. M., and White, E. C., *J. Am. Med. Assn.*, 1925, **84**, 1112.

produced a further decrease in food intake. The bromsulphalein retention increased in all 3 dogs, and the serum phosphatase increased in 2 of the 3 dogs to 8 to 10 units/100 cc. Liver biopsies taken during the 32nd week showed a considerable amount of fat in all 3 B complex deficient dogs. Neither the inanition control nor the normal control dogs showed abnormal changes in liver function or fatty changes in the biopsy.

At the end of the 33rd week each B complex deficient dog was given 4 g of type 3 yeast extract per day and the food intake was limited to 22 calories/sq. meter/hour, so that protein and total caloric intake was still below normal. One of the 3 dogs died during this period but the other 2 dogs showed a rapid return of bromsulphalein retention and serum phosphatase to normal. Liver sections at the 42nd week showed either a definite decrease in fat or complete return of the fatty change to normal.

Summary. A chronic vitamin B complex deficiency in dogs on a 41% casein diet produced a marked decrease in voluntary food intake, but liver function tests remained normal and liver sections showed no increase in fat. A similar chronic B complex deficiency using dogs on a 20% casein diet produced an abnormal liver function and fatty changes in liver biopsy specimens. These findings were absent in the inanition and normal control dogs. Therapy with yeast extract restored the liver function to normal and the fatty change in the liver decreased, even though protein and caloric intake were restricted.

15327

Role Played by Leucocytes, Platelets and Plasma Trypsin in Peptone Shock in the Dog.

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We have previously studied the mechanism leading to the discharge of histamine and heparin from the liver in an anaphylaxis-like condition produced in dogs by the injection of extracts from *Ascaris lumbricoides*.[1,2] On the basis of *in vitro* and *in vivo* experiments, performed in 84 dogs, we have concluded that the discharge of those substances constitutes the last step in a complex chain of reactions involving: (1) clumping of leucocytes and platelets which will form micro-thrombi inside the liver capillaries and small vessels; (2) disintegration of platelets (as shown in Giemsa stained smears from fragments of the liver) and also, in a less degree, of leucocytes in intimate contact with liver cells; (3) probable activation of a proteolytic enzyme, since platelets contain a kinase for plasma trypsin,[3] and (4) the release of histamine and heparin by activated trypsin, since this enzyme is able to accomplish both effects.[4,5]

All the steps of this chain of enzymatic and pharmacological reactions have been properly demonstrated, except activation of plasma trypsin during severe anaphylactic shock. When we attempted to estimate free trypsin in the plasma of animals, before and

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* From the Adolfo Lutz Institute, São Paulo, Brazil.

[1] Rocha e Silva, M., and Graña, A., *Arch. Surg.*, in press.

[2] Rocha e Silva, M., Porto, A., and Andrade, S. O., *Arch. Surg.*, in press.

[3] Iyengar, N. K., *Indian J. Med. Res.*, 1942, **30**, 467.

[4] Rocha e Silva, M., *Arch. f. exp. Path. und Pharmak.*, 1940, **194**, 335.

[5] Rocha e Silva, M., and Dragstedt, C. A., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 152.

TABLE 1.

Variations in Prothrombin Time, Histamine, Platelets and Leucocytes After the Injection of Peptone in the Dog.

N° of the dog	Wt of the dog	Intensity of the shock	Prothrombin time			Histamine (y/ml)			Platelets (ml/100 ml)			Leucocytes (1000/mm³)		
			Before	Max.	Final	Before	Max.	Final	Before	Min.	Final	Before	Min.	Final
P														
36	9	++++	12″	120″	00	.03	.87	.21	.45	.0	.10	25.0	3.0	5.2
40	4	++++	9	00	00	.06	.53	1.09	.27	.0	.0	6.7	0.4	1.6
58	14	++++	13	35	17″	.05	.58	.41	.70	.0	.05	12.9	2.6	2.9
51	9	+++	10	80	55	.16	.50	.11	.48	.0	.08	8.9	2.8	4.6
43	13	+++	15	35	240	.01	.15	.11	.20	.0	.12	5.7	1.8	2.3
50	10	+++	11	00	35	.11	1.00	.09	.22	.07	.12	5.0	1.4	2.1
59	7	++	11	14	13	.06	.41	.06	.45	.08	.35	9.3	2.8	6.4
44	15	++	11	11	10	.12	.15	.12	.65	.03	.42	12.1	2.4	13.3
47	22	++	9	9	8	.10	.25	.20	.90	.05	.49	18.3	5.7	13.8
33	5.5	++	9	9	9	.04	.11	.03	.35	.05	.25	17.3	3.3	8.2
52	13	++	11	22	14	.08	.35	.15	.65	.0	.40	15.9	2.9	19.2
37	9	++	10	12	9	.05	.23	.13	.50	.05	.45	14.7	6.4	8.5
,,	9	+	10	10	10	.13	.17	.11	.45	.12	.45	8.5	8.3	9.4
39	8.5	+	14	13	13	.03	.19	.06	.70	.25	.35	13.5	6.3	10.3

Note: The animals 40P and 50P received 10 mg of heparin per kilo of body weight, before the injection of peptone. As seen in the table, this heparin injection did not prevent the drop in platelets and leucocytes. Upon the blood pressure, the effect if any was rather an aggravating one.

Legend: ++++, animal died, 20′ to 60′ without recovery; +++, incomplete recovery, without return to normal level; ++, moderate fall in carotid blood pressure and return to normal in 20 to 30 min; +, fall in carotid blood pressure and return to normal level in <10 or 12 min.

after the onset of the anaphylactic reaction, the only effect we have been able to detect was a rather drastic reduction in both free and total trypsin. We have interpreted this finding by assuming that the heparin which appears in blood stream would instantaneously block the action of activated trypsin, since heparin was shown in separate experiments to antagonize plasma trypsin.[6] That this interpretation was correct, has been suggested by Jaques[7] who has observed that addition of protamine to blood samples collected at the height of the anaphylactic shock brings about coagulation and, in many instances, a quick redissolution of the coagulum. Since fibrinolysis strongly suggests activation of plasma trypsin,[8,9] this fact and those here presented constitute the demonstration that this enzyme might be concerned in the onset of anaphylactic and peptone shock.

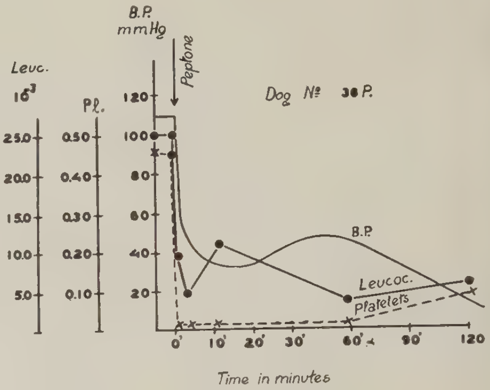

Fig. 1.

Dog 36P. Fatal shock. Variations in leucocytes, platelets and carotid blood pressure. Note that those variables went quickly to a very low level and remained so until death.

Material and Methods. The peptone used in those experiments was a proteose-peptone (Difco) containing small quantities of histamine, which amount was subtracted from the total, in experiments of liver perfusion; in the *in vivo* experiments, the histamine contained in the peptone injected was considered of minor significance for the final computation of the results. The heparin used was a Connaught preparation, containing 110 units per mg. The dogs were anesthetized with morphine (10 mg per kilo) plus Dial (Ciba) in the dose of 0.16 ml per kilo of body weight. When necessary, ether was used as a supplement. The *in vivo* experiments were performed by taking a tracing of the carotid blood pressure and collecting several blood samples before and at varying intervals after the injection of peptone. The samples were destined: (a) for a duplicate estimation of histamine by Code's method;[10] (b) for determination of prothrombin time by Quick's one stage method[11] and fibrinogen; (c) for leucocyte counting; (d) for estimation of platelets volume by the method of the Van Allen's thrombocytocrit;[12] (e) for estimation of heparin by the protamine test, following the indications of Jaques and Waters.[13] Data concerning fibrinogen are analyzed in a separate publication. Fibrinolysis was observed in the samples which clotted after addition of protamine, and the data indicated in the text refer to a period of 24 hours of observation. Perfusion experiments upon the isolated liver were performed according to the technic previously described.[1,2]

Results. In Table I and Fig. 1 and 2 are indicated the variations in prothrombin time, histamine, leucocytes and platelets in 13 dogs submitted to peptone shock. At least 10 samples were taken at several intervals before and after the injection of 300 mg of peptone per kilo of body weight. From this considerable mass of data, those values were selected to show the initial level, the maximum or the minimum for each variable and also the final value, indicating the capacity of each animal to recover from initial changes. There was always a sharp decrease in leucocytes and platelets immediately after

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[6] Rocha e Silva, M., and Andrade, S. O., *Science*, 1945, **102**, 670.

[7] Jaques, L. B., personal communication.

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[10] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

[11] Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, 1942, C. C. Thomas, Springfield.

[12] Van Allen, C. M., *J. Lab. Clin. Med.*, 1926, **12**, 282.

[13] Jaques, L. B., and Waters, E. T., *J. Physiol.*, 1941, **99**, 454.

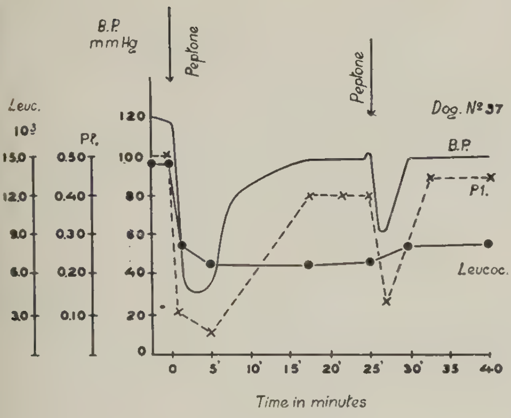

Fig. 2.

Dog 37P. A first injection of peptone produced profound drop in blood pressure but a recovery almost to normal, after 20′. A second injection produced a smaller effect and a quicker recovery. Platelets accompanied the curve of blood pressure, in a quite remarkable way.

the injection of the peptone, the minimum being attained, as a rule, after 3 to 5 minutes following the injection. The data for prothrombin time are indicated as a rough measure of the discharge of antithrombin (heparin), since we have observed a certain parallelism between increases in prothrombin time and the amounts of heparin present. Additional information concerning the discharge of heparin in 4 of the animals employed can be obtained in Fig. 3, in which the results given by protamine test are indicated.

In cases of slight (+) or moderate (++) shock, the return of leucocytes and platelets was very rapid, accompanying the return to normal of carotid blood pressure. In those cases in which the shock was maximal, as carotid blood pressure fell rapidly to zero, platelets were down for the duration of the shock and did not return until death. Also the discharge of histamine and heparin was maximal in those cases. In the case of dog 51P, the shock was particularly severe, with appreciable discharge of histamine and heparin, the blood pressure was maintained at a low level for a long while and only slowly did incomplete recovery occur. As indicated in the table, platelets fell considerably and remained down at the end of the experiment. Of course, we cannot establish an absolutely perfect parallelism between recovery and the capacity for platelets and leucocytes to return to their initial level. In the first place, when platelets are initially high, a partial return would not mean that the elements were destroyed somewhere in the body, since they can be retained in some unspecified organ structures, as occurs when one injects glycogen or any platelet-leucocyte agglutinating substance.[14] On the other hand, even when the leucocyte-platelet counts fully recover, there might occur some platelet destruction, since the returning elements might derive from previous depots in tissues which might not be concerned in the reaction. But, if one considers extreme cases, the parallelism indicated in Table I and Fig. 1 and 2, is striking enough to permit the conclusion that platelets and leucocytes take part in the mechanism of production of peptone shock.

Very important is the question as to where white elements segregate during peptone shock. From the perfusion experiments presented in another section of this paper it became very clear that the liver is able to retain those elements. But also *in vivo,* we have indications that those elements are concentrated in the portal circulation, since in many cases in which samples were taken at the trunk of the portal vein immediately after or before a similar sample was taken from femoral vein or inferior vena cava, leucocytes and platelets were enormously increased in the portal vein blood and in most cases there was a moderate but significant difference between those counts in portal and peripheral blood. In the case of dog 47P, for instance, the animal died with a leucocyte count of 4,500 per cu mm and a platelet volume of 0.10%, in a sample taken at inferior vena cava, while a sample taken simultaneously in the trunk of the portal vein showed a leucocyte count of 49,700 per cu mm and 0.50% of platelets. Another striking result was obtained in dog 58P, which died in 17 minutes, with leucocyte count of 1,600 and a platelet volume of 0.05%, while a simultaneous sample taken at the trunk of the portal vein showed 11,000 leucocytes

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[14] Rocha e Silva, M., Graña, A., and Porto, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 57.

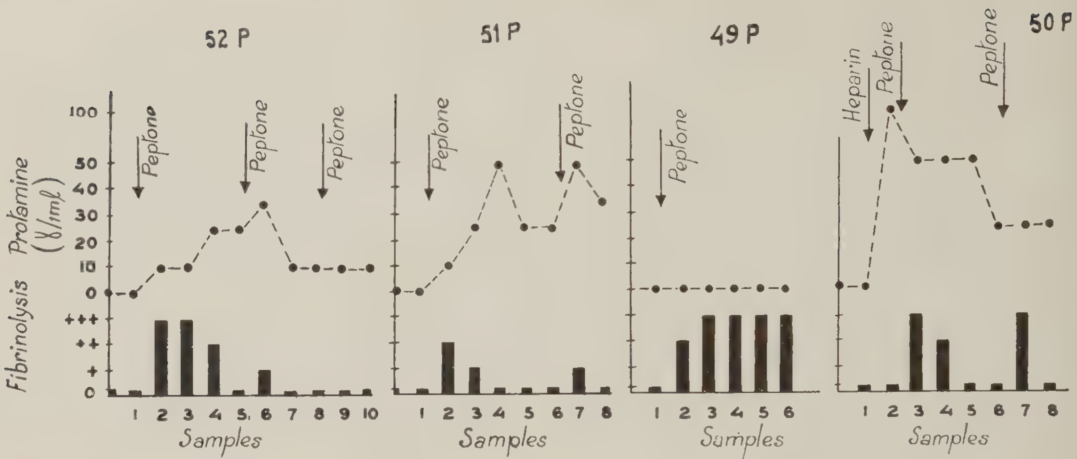

Fig. 3.

Fibrinolysis and heparin discharge during peptone shock. The curves indicate the optimum amount of protamine to counteract the effect of the heparin present in samples taken at different stages of the shock. The indicated fibrinolysis was observed in the samples which clotted after addition of the optimum or higher amounts of protamine.

per cu mm and 0.90% of platelets. Similar results were obtained in quite a few cases and are definitely in accordance with the idea that the liver behaves like a filter for the white blood elements, during peptone shock.

The data presented in Table I show a definite relationship between the discharge of histamine and of anti-coagulant and the severity of the shock. Hence, fall in carotid blood pressure, drop in leucocytes and platelet volume, discharge of histamine and heparin, as well as the gross appearance of the liver by direct inspection, constitute the variables that must be considered in describing anaphylactic and peptone shock in the dog. All of them appear to run quite parallel and this fact affords considerable evidence pointing to the idea that they are fundamentally interconnected. Besides those 6 variables, we have to consider hemorrhages to the intestinal mucosa, leading to bloody diarrhea, and activation of plasma trypsin, as indicated by the observation of fibrinolysis occurring after addition of protamine, as will be considered in the next paragraph. We now have indications that the occurrence of hemorrhages to the intestinal mucosa might also be a consequence of the activation of a tryptic enzyme, since trypsin given to normal or heparinized dogs, can produce hemorrhages in the intestinal tract, and this effect seems to depend upon a direct effect of the enzyme upon the vessels of that region, without very much relationship to the stasis and drop in carotid blood pressure.

Activation of plasma trypsin in peptone shock. As mentioned previously, estimation of plasma trypsin during anaphylactic shock in the dog showed that the changes were exactly the reverse of those we might expect from the assumption that disintegrated blood elements might activate this enzyme inside the liver parenchyme. There was consistently a drop in both free and total trypsin, somewhat correlated to the severity of the shock. This was interpreted as resulting from the discharge of heparin or any anti-tryptic heparin-like substance. In peptone shock, however, we had frequently observed many intriguing phenomena which could only be interpreted by assuming that plasma trypsin is actually activated. Sometimes, after the injection of peptone, the blood clotted very quickly (1′ to 2′) and in one instance it even clotted while pipetting into the tubes containing protamine (dog 49P); after a short while the clots redissolved. While doing those experiments, I received a note from Dr. L. B. Jaques, with the illuminating statement that in his experiments with the protamine test in anaphylactic shock, there frequently occurred a more or less extensive fibrinolysis in samples which clotted by adding protamine in suitable amounts. Therefore we decided to make systematic observations upon this phenomenon in peptone shock, and typical results are presented in Fig. 3. When the animals received 2 or more injections of peptone, it was observed that fibrinolysis is maximal after the first injection, moderate after the second injection and absent after the third injection. It is obvious that this desensitizing effect occurring also with fibrinolysis, strongly suggests that the latter is concerned with the very mechanism of the shock. In control experiments, the *in vitro* addition of large amounts of peptone directly to blood samples failed to produce any fibrinolytic effect.

In discussing the results obtained with this "fibrinolytic protamine test," we might recall that Nolf[15] had shown fibrinolysis occurring after peptone injection in the dog in those cases in which the liver was removed from circulation, or the circulation restricted to the upper part of the body, by ligating the inferior vena cava and thoracic aorta just above the diaphragm. Since heparin is discharged from the liver, it appears quite evident that shunting off the liver from circulation would bring experimental conditions for production of fibrinolysis quite similar to those obtained *in vitro* by adding an excess of protamine. Those facts substantiate the theory that activated plasma trypsin might constitute a final mediator for the discharge of histamine and heparin from liver cells, since that enzyme is able to produce both effects, not only *in vivo,* but also in experiments of liver perfusion.[2]

Experiments of liver perfusion with peptone. In the previous experiments performed with ascaris extracts it became apparent that the simple contact of the material with liver cells, when Tyrode solution was used as perfusing fluid, is unable to discharge histamine and heparin. When total citrated blood was used, there occurred considerable retention of leucocytes and platelets and, in one case, the discharge of histamine and anti-coagulant was large enough to afford the conclusion that blood contains all the elements which are necessary to produce that discharge. Quite similar results were obtained by perfusing dogs' liver with peptone. When Tyrode solution was used, even large amounts of peptone were unable to produce any reduction in the perfusing flow or increase in the size of the organ. Also histamine and heparin did not appear in the perfusates, even after 30 to 40 minutes of perfusion with Tyrode + peptone. However, when total heparinized blood containing 1 to 3 g of peptone was substituted for the Tyrode solution, immediately after the first portion of blood had passed, the flow was drastically reduced almost to zero (only a few drops passed, from an initial velocity of 80 to 100 ml per minute) and the organ showed enormous increase in volume, almost doubling in a few minutes. Counting of leucocytes and platelets before and after the passage through the organ showed enormous reductions. In the case of the dog 60P, for instance, the platelets that were very high (1.15%) at the beginning, dropped to 0.20 and 0.05% immediately after addition of peptone and passage through the liver; at the same time the leucocytes dropped from an initial level of 10,900 per cu mm to 1,000 and 900 per cu mm and those counts remained low for the duration of the experiment. Also the perfusion rate fell to a very low level (a few cu mm per minute) so that the perfusion could not proceed, since the organ was cold and dark, indicating a complete lack of irrigation by the perfusing fluid. In the drainage fluid (obtained by making sharp incisions in the organ, after stoppage of the perfusion) the leucocytes rose again to a high level (17,950 per cu mm) and the platelets volume went up to 0.85%. As concerns the appearance of histamine and heparin in the perfusates, when total blood was used as vehicle, we have to emphasize that the amounts never were as high as should be expected from *in vivo* experiments. Also fibrinolysis occurring in the perfusates could be observed only occasionally and was not as striking as in *in vivo* experiment. From those facts we might conclude either that some extra-hepatic factor contributes to rein-force activation of plasma trypsin as it occurs *in vivo,* or that the conditions of the perfusion experiments only exceptionally reproduce the conditions of the phenomenon as it occurs *in vivo*. Although those possibilities will be considered in future experiments, we can draw certain conclusions from the perfusion experiments concerning the mechanism of the preliminary phases of the shock, namely constriction of hepatic veins and retention of leucocytes and platelets inside the organ, these constituting the main factors for the enlargement of the organ and stasis in portal region. The importance of this retention was further proved in those experiments in which Tyrode was used as perfusing fluid and also in 2 other experiments in which the blood used for perfusion was extremely poor in white blood elements. In those cases, there was very slight diminution of flow, in contrast with the almost complete stoppage of the circulation when the white blood elements were high enough in the perfusing heparinized blood.

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[15] Nolf, P., *Medicine,* 1938, **17**, 381.

Conclusions. From the results described in this paper, it appears evident that peptone produces shock in the dog by a mechanism very similar to that leading to anaphylactic shock. Participation of the white blood elements (leucocytes and platelets) in the discharge of histamine and heparin from liver cells is definitely suggested by the fact that the severity of the shock is closely connected with the incapacity of those blood elements to return to blood stream, and also by experiments with isolated liver, in which it became apparent that peptone is unable by itself to release those substances from liver cells, and that the presence and retention of blood elements (leucocytes and platelets) is an indispensable step leading to the discharge of those substances. That the final mediator for the release of these substances is activated plasma trypsin is strongly suggested by the fact that platelets contain a kinase for plasma trypsin and that there actually occurs activation of this enzyme, as shown by fibrinolysis occurring after the injection of peptone. Also the fact that fibrinolysis is maximal after the first injection of peptone, moderate after a second injection and minimal or absent after a third injection, constitutes strong evidence that activation of plasma trypsin is actually concerned in the mechanism of production of the shock and that desensitization which occurs in those kinds of shock is probably dependent upon exhaustion of the capacity for activation of plasma trypsin. Experiments upon those lines are being actively conducted in this laboratory.

We have to thank Dr. Wilson T. Beraldo, of the Faculty of Medicine of São Paulo, for help given in the last phases of this work, and Mr. Jayme Ferraz, who made leucocyte and platelet estimations.

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Heredity of Rh Blood Types.* V. Improved Nomenclature; Additional Family Studies with Special Reference to Hr.

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In preceding papers of this series[1-4] data have been presented on the heredity of the Rh blood types in a total of 197 families with 463 children. Only 2 apparent exceptions to Wiener's theory[5] of 6 major allelic genes were encountered, and in both cases

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* Aided by a grant from the United Hospital Fund of New York City.

[1] Wiener, A. S., Sonn, E. B., and Belkin, R. B., *J. Exp. Med.*, 1944, **79**, 235.

[2] Wiener, A. S., Davidsohn, I., and Potter, E. L., *J. Exp. Med.*, 1945, **81**, 63.

the children proved to be illegitimate.

In determining to which of the 8 Rh types an individual belongs, tests are made with antisera for factors Rh_0, Rh′ and Rh″ (Wiener[6]). Levine[7] was the first to point out that Rh-negative blood is not merely characterized by its failure to clump in anti-Rh sera, because such blood was agglutinated by an antiserum obtained by him from an Rh-positive mother of an erythroblastotic infant. The factor detected by Levine in Rh-negative blood seemed to be reciprocally related to Rh, and so he named this new factor Hr. In England, Race and Taylor[8] under similar circumstances encountered a serum which also agglutinated all Rh-negative bloods, but gave 80% positive reactions instead of 30% as reported by Levine. The British workers designated their factor as St after the first 2 letters of the patient's name. Wiener *et al.*[2] showed that Hr and St were identical and pointed out that the difference in the number of positive reactions was due to the low titer of Levine's serum which therefore failed to react with heterozygous bloods.

As Race *et al.*[9] have shown, the Hr (or St) factor is present in the agglutinogens determined by the genes Rh_2, Rh_0, *Rh″* and *rh* but absent from the agglutinogens determined by genes Rh_1 and *Rh′* (cf. Wiener[10]). Therefore, the original or standard Hr factor is related to factor Rh′ as M is related to N.[2,11] To indicate this fact, we[12,13] suggested to designate the standard Hr factor simply as Hr′. This latter designation will become increasingly desirable in the future, especially since Fisher[14] suggested that there are 3 factors in Rh-negative blood, each reciprocally related to one of the 3 Rh factors. To date we have studied 3 different Hr antisera and all of these (like the original Hr serum of Levine, and of Race *et al.*) corresponded to anti-Hr′ or standard anti-Hr. Mourant[15] has recently encountered a serum giving reactions corresponding to those predicted for anti-Hr″, but to date no serum has been found corresponding to anti-Hr_0.

Of the 3 theoretically possible anti-Hr sera, only anti-Hr′ (or standard anti-Hr) is generally available at present. Fortunately, standard anti-Hr serum is the most valuable because it makes possible the subdivision of the most common Rh type, type Rh_1. The use of anti-Hr serum together with the 3 anti-Rh sera enhances considerably the practical value and theoretical interest of heredity studies on the Rh blood types. Race *et al.*[16,17] and Stratton[18] have published family studies on the Rh blood types in which Hr tests were also performed.

The purpose of this report is to present our recent experiences in family studies on the hereditary transmission of the Rh blood types and the standard Hr factor. We shall take this opportunity also to introduce some improvements in the nomenclature made possible by recent advances in our knowledge of this subject.

Materials and Methods. The blood samples tested in our heredity studies were derived from clinical material sent to us for diagnostic Rh tests in cases where it was suspected that one or more of the children in a family had congenital hemolytic disease (erythroblastosis fetalis). This accounts for the abnormal distribution of the Rh blood

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[3] Sonn, E. B., and Wiener, A. S., *J. Hered.*, 1945, **36**, 301.

[4] Wiener, A. S., and Sonn, E. B., *J. Lab. and Clin. Med.*, 1945, **30**, 395.

[5] Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 316.

[6] Wiener, A. S., *Am. J. Clin. Path.*, 1945, **15**, 106.

[7] Levine, P., *J. Ped.*, 1943, **23**, 656.

[8] Race, R. R., and Taylor, G. L., *Nature*, 1943, **152**, 300.

[9] Race, R. R., Taylor, G. L., Cappell, D. T., and McFarlane, M. N., *Nature*, 1944, **153**, 52.

[10] Wiener, A. S., *J. Am. Med. Assn.*, 1945, **127**, 294.

[11] Levine, P., *Science*, 1945, **102**, 1.

[12] Wiener, A. S., and Sonn, E. B., *Ann. N. Y. Acad. Sci.*, in press.

[13] Wiener, A. S., *Science*, 1945, **102**, 479.

[14] Fisher, R. A., cited by Race, R. R., *Nature*, 1944, **153**, 771.

[15] Mourant, E., *Nature*, 1945, **155**, 542.

[16] Race, R. R., Taylor, G. L., Ikin, E. M., and Prior, A. M., *Ann. Eug.*, 1944, **12**, 206.

[17] Race, R. R., Taylor, G. L., Ikin, E. M., and Dobson, A. M., *Ann. Eug.*, 1945, **12**, 261.

[18] Stratton, F., *Ann. Eug.*, 1945, **12**, 250.

TABLE I.
The Rh Blood Types and Subtypes.*

Rh blood types	Reactions with sera†			Genotypes	Reactions with sera†		Rh subtypes	Distribution (%) among 645 Caucasians in N.Y.C.	
	Anti-Rh′	Anti-Rh″	Anti-Rh$_0$		Anti-Hr′	Anti-Hr″			
rh	Neg.	Neg.	Neg.	rr	Pos.	Pos.		12.9	
Rh′	Pos.	Neg.	Neg.	$R'R'$	Neg.	Pos.	Rh′Rh′	.01‡	0.9
				$R'r$	Pos.	Pos.	Rh′rh	0.9 ‡	
Rh″	Neg.	Pos.	Neg.	$R''R''$	Pos.	Neg.	Rh″Rh″	.004‡	0.5
				$R''r$	Pos.	Pos.	Rh″rh	0.5 ‡	
Rh′Rh″	Pos.	Pos.	Neg.	$R'R''$	Pos.	Pos.		.01‡	
rh$_0$	Neg.	Neg.	Pos.	r^0r^0 r^0r	Pos.	Pos.		1.9	
Rh$_1$ (Rh$'_0$)	Pos.	Neg.	Pos.	R^1R^1 R^1R'	Neg.	Pos.	Rh$_1$Rh$_1$	20.2	53.7
				R^1r R^1r^0 $R'r^0$	Pos.	Pos.	Rh$_1$rh	33.5	
Rh$_2$ (Rh$''_0$)	Neg.	Pos.	Pos.	R^2R^2 R^2R''	Pos.	Neg.	Rh$_2$Rh$_2$	3.1‡	15.3
				R^2r R^2r^0 $R''r^0$	Pos.	Pos.	Rh$_2$rh	12.2‡	
Rh$_1$Rh$_2$	Pos.	Pos.	Pos.	R^1R^2 $R'R^2$ R^1R''	Pos.	Pos.		14.4	
Others§								0.5	

* Does not include Rh$_z$ or the intermediate Rh blood types, or the reactions with the hypothetical serum anti-Hr$_0$.

† Anti-Rh$_0$ is the standard anti-Rh serum (85% positive); anti-Hr′ is the standard anti-Hr serum (80% positive).

‡ These frequencies were calculated on the basis of the 6 gene theory. All other frequencies given were actually observed. All 645 bloods were tested with anti-Rh′, anti-Rh″, anti-Rh$_0$ and anti-Hr′ sera; anti-Hr″ serum was not available to us.

§ There were 3 bloods giving atypical reactions; namely, one of type Rh$_1$Rh$_z$ and 2 intermediates.

types among the parents, in particular the high percentage of Rh-negative mothers. A random series of blood samples from male professional blood donors was also examined, for use as an index of the distribution of the Rh blood types among Caucasian individuals in New York City. With only a few exceptions, all blood samples were obtained by venipuncture; 5 cc of blood were mixed with powdered potassium and ammonium oxalate in a small test tube. The tubes containing the oxalate powder were prepared in the same way as for the Wintrobe-Landsberg[19] sedimentation test.

The tests were carried out within 24 to 48 hours after the blood was drawn, the blood samples being kept in the refrigerator in the interim. Blood group, subgroup and M-N tests were performed on every specimen by the well-slide method as described elsewhere.[20] For the Rh and Hr tests, all blood suspensions were washed once with saline solution and the tests were set up in small tubes as already described.[1,21]

Nomenclature. The recent work with anti-Hr′ and anti-Hr″ sera makes desirable certain changes and improvements in the nomenclature. Naturally such changes should be kept to an absolute minimum, and it must be emphasized that the recent results have merely added to our knowledge and not changed our previous conclusions. The improved nomenclature is presented in Table I.

Firstly, in regard to the Rh blood types, it is proposed to substitute the simple desig-

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[19] Wintrobe, M. M., and Landsberg, J. W., *Am. J. Med. Sci.*, 1935, **189**, 102.

[20] Wiener, A. S., *Blood Groups and Transfusion*, 3rd ed., C. C. Thomas, Springfield, Ill., 1945.

[21] Wiener, A. S., Zepeda, J. P., Sonn, E. B., and Polivka, H. R., *J. Exp. Med.*, 1945, **81**, 559.

TABLE II.
List of Family Material.

Family No.	Father	Mother	Children	
1.	$OMRh_2Hr+$	$A_1MRh-Hr+$	A_1MRh_2Hr+ ♀	
2.	$ONRh_1Hr-$	$A_1MRh-Hr+$	$OMNRh_1Hr+$ ♀	
3.	A_1NRh_1Hr+	$OMNRh_1Hr+$	$ONRh_1Hr+$ ♀	$ANRh_1Hr-$ ♂
4.	$OMNRh_1Hr-$	$A_1MRh-Hr+$	A_1MNRh_1Hr+ ♂ $OMNRh_1Hr+$ ♀	A_1MNRh_1Hr+ ♀ $OMRh_1Hr+$ ♂
5.	$OMRh_1Rh_2Hr+$	$BMNRh-Hr+$	$BMRh_2Hr+$	
6.	$OMNRh_1Hr-$	$OMRh'Hr+$	$OMRh_1Hr+$	
7.	$BMNRh_1Hr-$	$BMNRh-Hr+$	$BMNRh_1Hr+$ ♀	$BNRh_1Hr+$ ♂
8.	A_1MNRh_1Hr-	$ONRh_1Hr+$	A_1MNRh_1Hr+	
9.	$OMNRh_1Hr+$	$OMRh-Hr+$	$OMRh_1Hr+$ ♂	
10.	A_1BMNRh_1Hr-	$A_1MNRb-Hr+$	A_1NRh_1Hr+ ♂	
11.	A_1MRh_1Hr-	$A_1MNRh-Hr+$	A_1MRh_1Hr+ ♀	A_1MRh_1Hr+ ♀
12.	A_1BNRh_1Hr+	$OMNRh'Hr+$	$BMNRh'Hr+$ ♂	
13.	$OMRh_2Hr+$	$A_2MNRh-Hr+$	A_2MNRh_0Hr+ ♂ $OMNRh_2Hr+$ ♂	$OMNRh_2Hr+$ ♀
14.	A_2BMNRh_1Hr+	$ONRh_1Rh_2Hr+$	$BMNRh_2Hr+$	
15.	$OMNRh_1Rh_2Hr+$	$A_1BMNRh-Hr+$	$BMRh_1Hr+$ ♂ $BMRh_2Hr+$ ♂	$BMNRh_2Hr+$ ♀
16.	$OMNRh_1Hr-$	$A_2MRh-Hr+$	A_2MRh_1Hr+ ♀	
17.	$A_1MRh_1Rh_2Hr+$	$A_2MNRh-Hr+$	A_1MNRh_1Hr+ ♀	A_1MNRh_1Hr+ ♂
18.	$OMNRh_2Hr+$	$OMRh_1Rh_2Hr+$	$OMNRh_2Hr+$ ♀	$OMRh_1Hr+$
19.	$A_1NRh_1Rh_2Hr+$	$OMNRh_1Rh_2Hr+$	$OMNRh_1Rh_2Hr+$ ♀	
20.	$A_1MNRh'Hr+$	A_1MNRh_1Hr-	A_2MNRh_1Hr-	
21.	$A_1BNRh_1Rh_2Hr+$	$OMRh_2Hr+$	$BMNRh_1Hr+$	
22.	$OMRh_1Hr-$	A_1NRh_1Hr-	$OMNRh_1Hr-$ ♂	
23.	A_1NRh_0Hr+	$OMNRh_1Rh_2Hr+$	A_1NRh_1Hr+ ♀	
24.	$OMNRh_1Rh_2Hr+$	A_1MRh_1Hr+	$OMRh_1Rh_2Hr+$ ♂ A_1MRh_1Hr- ♂	$A_1MNRh_1Rh_2Hr+$ ♂
25.	A_2NRh_1Hr+	$A_1BMNRh-Hr+$	A_1MNRh_1Hr+ ♀	$BNRh_1Hr+$ ♂
26.	$BNRh-Hr+$	$OMNRh_1Hr+$	$BMNRh-Hr+$ ♂	$BNRh_1Hr+$ ♂
27.	$BNRh_1Hr+$	A_1NRh_1Hr+	$ONRh_1Hr-$ ♂	$ONRh_1Hr+$ ♀
28.	$OMNRh_1Hr-$	$OMRh_1Hr+$	$OMRh_1Hr-$	
29.	$A_1MNRh_1Rh_2Hr+$	$A_1MNRh-Hr+$	A_1NRh_2Hr+ ♀	
30.	A_1MRh_1Hr-	A_1MNRh_1Hr+	A_1MRh_1Hr+ ♀	
31.	A_1MNRh_2Hr+	A_1MNRh_1Hr+	$ONRh-Hr+$ ♂	
32.	$OMRh_1Hr+$	$A_1MNRh-Hr+$	A_1MRh_1Hr+ ♀	A_1MRh_1Hr+ ♂
33.	$A_1MRh_1Rh_2Hr+$	A_1MRh_1Hr+	A_1MRh_2Hr+ ♀	
34.	A_1MNRh_1Hr+	$OMRh_1Hr-$	$OMNRh_1Hr-$ ♀	ARh_1Hr+
35.	$OMNRh_1Hr+$	$OMNRh_1Hr+$	$OMNRh_1Hr+$ ♀	$OMNRh-Hr+$ ♀
36.	$OMNRh_1Hr-$	$BMNRh-Hr+$	$BNRh_1Hr+$ ♂	
37.	$A_2BMNRh_1Rh_2Hr+$	$A_1MNRh-Hr+$	A_1BMNRh_2Hr+ ♀	$BMNRh_1Hr+$ ♂
38.	$A_1MNRh_1Rh_2Hr+$	$A_1NRh-Hr+$	A_1MNRh_2Hr+ ♂	
39.*	$OMNRh_1Hr-$	$A_2BNRh-Hr+$	A_2BMNRh_1Hr+ ♀	A_2Rh_1Hr+ ♂
40.	A_2MNRh_1Hr+	$OMNRh_1Hr+$	$OMNRh_1Hr+$ ♂	$OMNRh_1Hr+$ ♂
41.	A_1MNRh_1Hr+	$ONRh_1Hr+$	A_1NRh_1Hr- ♀	
42.	$BMNRh_1Rh_2Hr+$	$ONRh_1Hr-$	$BMNRh_1Hr-$ ♂	
43.	$A_1BMNRh-Hr+$	$OMRh-Hr+$	$BMNRh-Hr+$	
44.	$A_1MNRh-Hr+$	$OMRh-Hr+$	$OMRh-Hr+$ ♂	
45.*	$BMNRh_1Hr+$	$A_2MRh-Hr+$	$A_2MRh-Hr+$ ♀	A_2BMRh_0Hr+ ♀
46.	$OMNRh_1Rh_2Hr+$	$OMRh-Hr+$	$OMRh_2Hr+$ ♀	$OMRh_1Hr+$ ♀
47.	A_1MNRh_1Hr+	$ONRh-Hr+$	$A_1NRh-Hr+$ ♀	$ONRh-Hr+$
48.	$BMRh_1Hr-$	$OMNRh-Hr+$	$BMNRh_1Hr+$ ♂	
49.	$OMNRh_1Hr-$	$OMNRh-Hr+$	$OMNRh_1Hr+$	
50.	$OMNRh_2Hr+$	$A_1MRh_1Rh_2Hr+$	$OMRh_2Hr+$ ♂	
51.	$OMRh_1Hr+$	$A_1MNRh-Hr+$	$OMNRh_1Hr+$ ♀	$OMNRh_1Hr+$ ♂
52.	$OMNRh_1Hr+$	A_1NRh_1Hr+	$ONRh_1Hr+$ ♂	
53.	A_2MRh_2Hr+	$A_{1,2}MNRh-Hr+$	$A_2MNRh-Hr+$ ♂	$OMNRh_2Hr+$ ♀
54.	$OMRh_1Hr+$	A_2BMNRh_1Hr+	$A_2MRh-Hr+$ ♂	
55.	$BMRh_1Hr+$	$OMNRh_1Hr+$	$BMRh_1Hr+$ ♀	$OMRh_1Hr+$ ♀
56.	A_2MNRh_2Hr+	$OMNRh_1Hr+$	$A_2MRh-Hr+$ ♀ $ONRh_1Hr+$ ♀	$A_2MNRh-Hr+$ ♀
57.	$OMNRh_1Hr+$	$OMNRh-Hr+$	$OMNRh_1Hr+$ ♂	$OMNRh-Hr+$ ♀
58.	A_2MNRh_2Hr+	$A_1MRh'Hr+$	A_1MRh_2Hr+ ♂	
59.	$OMRh_1Rh_2Hr+$	$A_1MNRh-Hr+$	A_1MRh_2Hr+ ♂	

TABLE II. (Continued).

Family No.	Father	Mother	Children	
60.	$BMRh_1Hr+$	ONRh— Hr+	$OMNRh_1Hr+$ ♂	$OMNRh_1Hr+$ ♀
61.	A_1MNRh_1Hr+	A_1BMNRh— Hr+	A_1BMNRh— Hr+	
62.	$OMRh_1Hr$—	$BMRh_1Hr$—	$OMRh_1Hr$— ♂	
63.	$OMRh_1Hr$—	OMRh— Hr+	$OMRh_1Hr+$ ♂	$OMRh_1Hr+$ ♀
			$OMRh_1Hr+$ ♀	$OMRh_1Hr+$ ♀
			$OMRh_1Hr+$ ♀	$OMRh_1Hr+$ ♂
			$OMRh_1Hr+$ ♀	
64.	$OMNRh_1Rh_2Hr+$	A_2MNRh— Hr+	$OMNRh_2Hr+$ ♂	A_2MNRh_2Hr+ ♀
65.	A_1MNRh_1Hr—	OMRh— Hr+	A_1MRh_1Hr+ ♂	
66.	A_1MNRh_2Hr+	A_1MNRh— Hr+	$OMNRh_2Hr+$ ♂	
67.	A_1NRh_1Hr+	$OMNRh_1Hr$—	A_1MNRh_1Hr— ♀	
68.	$BNRh_1Rh_2Hr+$	$ONRh_1Rh_2Hr+$	$BNRh_1Hr$— ♂	$ONRh_1Hr$— ♂
			$BNRh_2Hr+$ ♂	
69.	$BMRh_1Rh_2Hr+$	A_1NRh— Hr+	$OMNRh_2Hr+$ ♂	A_1BMNRh_1Hr+ ♂
70.	$OMNRh_1Rh_2Hr+$	A_1MNRh_1Hr+	A_1MRh_1Hr— ♂	$A_1MNRh_1Rh_2Hr+$ ♂
			$OMRh_1Rh_2Hr+$ ♂	
71.	$A_1NRh_1Rh_2Hr+$	$OMRh_1Hr+$	$AMNRh_1Hr$—	
72.	A_1NRh_1Hr+	$OMRh_1Hr$—	$OMNRh_1Hr+$ ♂	A_1MNRh_1Hr+
73.	$A_1BNRh_1Rh_2Hr+$	$OMRh_2Hr+$	$BMNRh_1Hr+$	
74.	$OMRh_1Rh_2Hr+$	A_1MRh— Hr+	A_1MRh_2Hr+ ♂	
75.	$OMNRh_2Hr+$	A_1MRh— Hr+	A_1MNRh_2Hr+ ♂	
76.	$ONRh_1Hr+$	$OMNRh_2Hr+$	$ONRh_2Hr+$ ♂	$OMNRh_1Hr+$ ♂
			$OMNRh_1Hr+$ ♂	
77.	$A_1MRh_1Rh_2Hr+$	A_1NRh_1Hr—	$OMNRh_1Rh_2Hr+$	
78.	$OMRh_0Hr+$	BMRh— Hr+	$OMRh_0Hr+$ ♂	
79.	$BNRh_1Hr+$	BMRh′Hr+	BMNRh′Hr— ♀	
80.	$OMRh_1Rh_2Hr+$	ONRh— Hr+	$OMNRh_2$ ♂	
81.	$OMNRh_1Hr+$	A_2MNRh—	A_2MRh— ♂	A_2MRh_1 ♀
			A_2MNRh_1 ♀	A_2MNRh_1 ♀
			ANRh— ♀	
82.	$ONRh_1Hr$—	A_1MNRh— Hr+	$OMNRh_1$ ♀	A_1MNRh_1 ♀
83.	$A_2MNRh_1Rh_2$	A_2MRh—	A_2MNRh_2Hr+ ♂	
84.	A_1MNRh_1Hr—	A_1MNRh— Hr+	$OMNRh_1$ ♀	A_1MRh_1Hr+ ♀
85.	$OMRh_1Rh_2$	A_1MNRh—	A_1MRh_1Hr+ ♂	A_1MRh_1Hr+ ♀
			A_2MRh_1Hr+ ♀	A_2MNRh_1Hr+ ♂
86.	$BMNRh_1Hr+$	$OMNRh_1Rh_2Hr+$	$BMNRh_1Hr$— ♂	$BMNRh_1$ ♀
87.	A_1MNRh_1Hr—	OMRh— Hr+	$A_1\ MNRh_1$ ♀	
88.	A_1NRh_1Hr+	$OMNRh_2$	A_1NRh_2 ♂	
89.	A_1NRh_2	$A_1MNRh_1Rh_2$	A_1MNRh_2 ♀	A_3NRh_1 ♂
90.	A_1MRh_1	OMNRh—	OMRh— ♂ †	A_2MRh_1 ♂ †
91.	$BMNRh_1$	AMRh—	$AMRh_0$	
92.‡	BMRh—	A_2BMRh—	BMRh— ♂	A_2BMRh— ♂
93.‡	A_2MRh_1	$BMRh_0$	A_2BMRh— ♀	
94.	$OMNRh_1Rh_2$	ONRh—	$ONRh_1$ ♀	
95.	$OMRh_1$	A_1MRh_2	OMRh—	
96.	A_1MRh—	$ONRh_1$	A_1MNRh—	
97.	$ONRh_1Rh_2$	OMNRh—	$OMNRh_1$ ♂	
98.	A_1MNRh_1	$OMRh_2$	$AMRh_1$	
99.	$OMRh_2$	A_1MRh—	A_1MRh_2 ♀	
100.	A_1MNRh_2	$A_{1,2}MRh_1$	A_1MRh_1 ♀	
101.	$BMNRh_1$	A_1BMRh—	$BMRh_1$ ♀	
102.	A_1MRh_1	A_2NRh_1	A_2MNRh_1 ♂	
103.	A_1MNRh_1	A_1NRh_1	A_1NRh_1 ♀	A_1MNRh_1
104.	$A_1MNRh_1Rh_2$	AMNRh—	A_1NRh_2	
105.	$OMRh_2$	OMRh—	$OMRh_2$ ♂	$OMRh_2$ ♀
			$OMRh_2$ ♂	
106.	A_1MRh_1	BMNRh—	$OMNRh_1$ ♂ §	$OMNRh_1$ ♂ §
107.	$BMRh_1Rh_2$	A_1MNRh_2	$BMRh_2$ ♀	
108.	$OMRh_1$	A_1MRh—	$OMRh_1$	
109.	A_1MNRh_1	A_1MNRh—	$OMNRh_1$ ♂	$OMRh_1$ ♀

* First child in families 39 and 45 from former marriage of wife.

† Stillborn twins.

‡ Daughter in family 93 same as mother in family 92.

§ Twins.

TABLE III.
Summary of Family Material of Table II.

Mating	No. of families	No. of children of types						Totals
		rh	Rh_1	Rh_2	Rh_1Rh_2	rh_0	Rh′	
rh × rh	3	4	0	0	0	0	0	4
rh × Rh_1	33	9	49	0	0	2	0	60
rh × Rh_2	7	1	0	10	0	1	0	12
rh × Rh_1Rh_2	17	0	12	15	0	0	0	27
rh × rh_0	1	0	0	0	0	1	0	1
Rh_1 × Rh_1	18	2	24	0	0	0	0	26
Rh_1 × Rh_2	7	4	5	2	0	0	0	11
Rh_1 × Rh_1Rh_2	8	0	6	2	5	0	0	13
Rh_1 × rh_0	1	1	0	0	0	0	0	1
Rh_1 × Rh′	4	0	2	0	0	0	2	4
Rh_2 × Rh_1Rh_2	6	0	4	4	0	0	0	8
Rh_1Rh_2 × Rh_1Rh_2	2	0	2	1	1	0	0	4
Rh_1Rh_2 × rh_0	1	0	1	0	0	0	0	1
Rh_2 × Rh′	1	0	0	1	0	0	0	1
Totals	109	21	105	35	6	4	2	173

nation "rh"† for the more cumbersome "Rh negative." The small "r" is used to indicate that type rh is recessive to the other Rh blood types. For type rh_0 the capital letter is no longer used, in order to indicate that this type has the same position in relation to types Rh_1, Rh_2 and Rh_1Rh_2 as type rh has in relation to types Rh′, Rh″ and Rh′Rh″.

When the reactions of the anti-Hr′ serum is taken into account, types Rh_1 and Rh′ are both subdivided into 2 types. Only bloods with a double dose of the Rh′ factor are Hr′ negative, and to indicate this Hr′-negative blood of type Rh_1 is designated as subtype Rh_1Rh_1 and the corresponding subtype of type Rh′ is designated Rh′Rh′. Blood of type Rh_1 reacting with Hr′ antiserum is designated as subtype Rh_1rh; the corresponding subtype of type Rh′ is designated Rh′rh. With the aid of anti-Hr″ serum similar subtypes can be distinguished in types Rh_2 and Rh″. The advantages of the names for the subtypes proposed by us are their simplicity and the lack of ambiguity so that they cannot be confused with the names of the 8 Rh blood types or the genotypes.

Our previous designations[22] for the 6 standard Rh genes have been criticized because the use of the subscripts for some of the allelic genes does not conform with the practice of geneticists. Another possible objection is that the use of the same symbol for the gene and corresponding agglutinogen could cause uncertainty in some cases as to whether the genotype or phenotype was intended, even though italics is used for the gene symbols. These objections are both met by the new designations of the genes in which only superscripts are used and the letter "h" has been dropped as suggested by British workers.‡ Thus, the new designations have the advantages of extreme simplicity, lack of ambiguity and conformity with genetic nomenclature.

The omission of the "h" from the designations of the genes makes it possible to use without danger of confusion the symbols *Rh* and *rh* when discussing the heredity of the Rh factor in general (factor Rh_0); for example, when presenting the theory of Land-

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† In giving the names of the Rh blood types verbally, it is not necessary to use the expressions "large Rh" or "small Rh" as the case may be, but merely to say "Rh," because the distinguishing subscripts and superscripts for the various types remove any danger of ambiguity.

[22] Wiener, A. S., *Science*, 1944, **99**, 532.

‡ The new simplified gene symbols, except for r^0, are the same as those approved by the majority of the members of the American Committee on Nomenclature of Blood Group Genes (W. C. Boyd, chairman; Th. Dobzhansky, P. Levine, L. H. Snyder, H. H. Strandskov and A. S. Wiener) as well as the British Committee (R. A. Fisher, E. B. Foord, K. Mather and R. R. Race).

TABLE IV.
Summary of Authors' Family Studies to Date.

Mating	No. of families	No. of children of types							Totals
		rh	Rh_1	Rh_2	Rh_1Rh_2	rh_0	Rh′	Rh″	
rh × rh	7	18	0	0	0	0	0	0	18
rh × Rh_1	82	34	122	0	0	9	0	0	165
rh × Rh_2	23	11	0	30	0	1	0	0	42
rh × Rh_1Rh_2	32	(1)	30	29	0	0	0	0	60
rh × rh_0	4	3	0	0	0	4	0	0	7
rh × Rh′	2	1	0	(1)	0	0	1	0	3
rh × Rh″	1	1	0	0	0	0	0	5	6
Rh_1 × Rh_1	44	7	86	0	0	4	1	0	98
Rh_1 × Rh_2	28	10	20	9	18	0	2	0	59
Rh_1 × Rh_1Rh_2	35	0	52	10	30	0	0	0	92
Rh_1 × rh_0	2	2	0	0	0	0	0	0	2
Rh_1 × Rh′	7	0	5	0	0	0	2	0	7
Rh_1 × Rh″	4	1	2	0	3	0	0	0	6
Rh_2 × Rh_2	2	1	0	4	0	0	0	0	5
Rh_2 × Rh_1Rh_2	12	0	6	12	8	0	0	0	26
Rh_2 × rh_0	2	0	0	1	0	4	0	0	5
Rh_2 × Rh′	1	0	0	1	0	0	0	0	1
Rh_1Rh_2 × Rh_1Rh_2	6	0	3	2	4	0	0	0	9
Rh_1Rh_2 × rh_0	3	0	2	1	0	0	0	0	3
Rh_1Rh_2 × Rh′	2	0	1	1	2	0	0	0	4
rh_0 × Rh′	1	0	0	0	0	1	1	0	2
Totals	300	90	329	101	65	23	7	5	620

steiner and Wiener of the inheritance of the Rh factor as a simple Mendelian dominant. Similarly, when discussing the genetic relationship of factors Rh′ and Hr′, one may say, without danger of ambiguity, that these factors are transmitted by means of a pair of allelic genes *Rh′* and *Hr′* analogous to the genes *M* and *N*. Similarly, if tests are made only with anti-Rh″ and anti-Hr″ sera, 2 factors and 3 types would be distinguished which are inherited by a pair of allelic genes *Rh″* and *Hr″*.

Finally, the simplified nomenclature proposed here avoids the introduction of any new symbols or letters, like C, D and E, which are entirely foreign to the scheme of the Rh blood types.

Results. Our list of family material is given in Table II.

First, it should be mentioned that there are no contradictions to the laws of inheritance of the A-B-O groups, A_1-A_2 subgroups or M-N types. It is of interest to note that one individual of subgroup A_3 was encountered in family No. 89.

In Table III we have summarized the Rh tests without taking into account the Hr reactions. In a total of 109 families with 173 children there is not a single exception to the theory of 6 allelic Rh genes. When these results are combined with our previously published findings, we have to date a total[§] of 300 families with 620 children tested for the 8 Rh blood types (cf. Table IV). As has already been mentioned, the 2 apparent contradictions to the theory were encountered in our previous studies and both proved to be due to illegitimacy.

In Table V are summarized the families in which Hr tests were carried out in addition to the Rh tests. It will be seen that when either or both parents belonged to type rh, rh_0, Rh_2 or Rh″, none of the children belonged to subtype Rh_1Rh_1 or type Rh′Rh″; on the other hand, when either or both parents belonged to subtype Rh_1Rh_1, none of the children belonged to type rh, rh_0, Rh_2 or Rh″. This confirms the theory that factors Rh′ and Hr′ are related to each other as M is to N. In 13 families rh × Rh_1Rh_1 all 24 children belonged to subtype Rh_1rh in conformity with the theoretical requirements;

[§] In Table IV, the Rh_1Rh_2 × Rh_1Rh_2 matings from our very first study[1] have been omitted because these appeared to contain some technical errors.

TABLE V.
Summary of Families on Which Hr Tests Were Done.

Mating	No. of families	No. of children of types (or subtypes)								Totals
		rh	Rh_1rh	Rh_1Rh_1	Rh_2	Rh_1Rh_2	rh_0	Rh'rh	Rh'Rh'	
rh × rh	2	2	0	0	0	0	0	0	0	2
rh × Rh_1rh	10	5	11	0	0	0	1	0	0	17
rh × Rh_1Rh_1	13	0	24	0	0	0	0	0	0	24
rh × Rh_2	5	1	0	0	6	0	1	0	0	8
rh × Rh_1Rh_2	11	0	6	0	12	0	0	0	0	18
rh × rh_0	1	0	0	0	0	0	1	0	0	1
Rh_1rh × Rh_1rh	8	2	7	3	0	0	0	0	0	12
Rh_1rh × Rh_1Rh_1	6	0	5	3	0	0	0	0	0	8
Rh_1Rh_1 × Rh_1Rh_1	2	0	0	2	0	0	0	0	0	2
Rh_1rh × Rh_2	3	3	3	0	1	0	0	0	0	7
Rh_1rh × Rh_1Rh_2	6	0	0	4	2	4	0	0	0	10
Rh_1Rh_1 × Rh_1Rh_2	2	0	0	1	0	1	0	0	0	2
Rh_1rh × Rh'rh	2	0	0	0	0	0	0	1	1	2
Rh_1Rh_1 × Rh'rh	2	0	1	1	0	0	0	0	0	2
Rh_2 × Rh_1Rh_2	4	0	3	0	2	0	0	0	0	5
Rh_2 × Rh'rh	1	0	0	0	1	0	0	0	0	1
Rh_1Rh_2 × Rh_1Rh_2	2	0	0	2	1	1	0	0	0	4
rh_0 × Rh_1Rh_2	1	0	1	0	0	0	0	0	0	1
Totals	81	13	61	16	25	6	3	1	1	126

on the other hand, in 10 matings rh × Rh_1rh with 17 children there were as many as 5 children of type rh and one child of type rh_0. The most unusual case is family No. 79. Here the father belonged to subtype Rh_1rh, the mother to subtype Rh'rh, while the daughter belonged to subtype Rh'Rh'. As shown in Table I, individuals of subtype Rh_1rh may belong to any one of the 3 possible genotypes, R^1r, R^1r^0 or $R'r^0$. In order for the couple in question to have a child of subtype Rh'Rh', it is evident that the father must belong to genotype $R'r^0$.

Another interesting case is family No. 45. The mother belonged to type rh, her husband to type Rh_1rh, the older daughter to type rh and the younger daughter to type rh_0. Obviously the husband could not be the father of both daughters so we concluded that the first child was probably the result of a former marriage. When we called the mother back for a private interview she told us that such was indeed the case and that she had not mentioned it before because she did not want to discuss the matter in the presence of her daughters.

Statistical Analysis. In Table I is given the distribution of the Rh blood types and subtypes in a series of 645 Caucasians in New York City. Since anti-Hr'' serum was not available to us, the percentages of the subtypes of types Rh_2 and Rh'' had to be computed on the basis of the 6-gene theory, but all the other frequencies listed were actually observed. In our experience to date we have only encountered a single blood of the rare type Rh'Rh'', and this was a blood sample sent to us by Dr. L. J. Unger for confirmation. It will be noted that one blood of the rare type Rh_1Rh_z and 2 bloods giving intermediate reactions were encountered in the present series. The blood of subtype Rh'Rh' from family No. 79 mentioned above is the only blood of this rare subtype that we encountered to date; no blood of this subtype occurred in our random series of 645 individuals.

The calculated gene frequencies based on the 645 random bloods, using the formulae given in a previous paper[1] are as follows: $r = 35.9\%$, $R' = 1.2\%$, $R'' = 0.6\%$, $r^0 = 2.5\%$, $R^1 = 44.9\%$, and $R^2 = 16.9\%$.

$$\text{Therefore, } D = 100 - \Sigma R = -2.0\%$$
$$P.E._D = \pm 0.8\%$$

Accordingly, the findings agree satisfactorily with the 6-gene theory. This may seem paradoxical because it is now known that there are more than 6 genes in the Rh allelic series.[23] The explanation is that the other genes are quite rare. The observed value for the frequency of type Rh_1Rh_1 (20.2%) agrees closely with the theoretically calculated value (20.0%) under the theory that factor Hr′ is related to factor Rh′ as M is related to N.

As has been pointed out[13] the Hr factor considerably increases the chances of exclusion in cases of disputed parentage. On Jan. 31, 1946, one of us (W.) testified in a case before the Court of Special Sessions of New York County, in which the defendant was excluded because he belonged to subtype Rh_1Rh_1 while the child belonged to type Rh_2. This case was tried before a very progressive Bench comprising Judges Nathan Perlman, John B. Flood and Irving Ben Cooper. Though this was the first time an Rh-Hr exclusion was presented in a courtroom, the defendant was acquitted, primarily on the basis of the blood test findings.

Summary. New additions to and further simplifications of the designations of Rh blood types, subtypes and genes are proposed in order to encourage the more general use of the Rh and Hr tests by medical men and geneticists.

Investigations on the hereditary transmission of the 8 Rh blood types in 300 families with 620 children yielded results in conformity with the theory of 6 major allelic genes.

Studies on the Hr factor in 81 families with 126 children and on a random series of 645 Caucasians in New York City fully support the theory that factors Rh′ and Hr′ are related to each other genetically and serologically like the agglutinogens M and N.

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[23] Wiener, A. S., *Science,* 1945, **100**, 595.

15329 P

Pathogenesis of Erythroblastosis Fetalis.*

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The present accepted theory of isoimmunization in pregnancy as the cause of congenital hemolytic disease does not explain the manifold clinical manifestations; nor why in the majority of cases no abnormal isoagglutinins can be detected in the maternal serum; nor why in some cases where the maternal serum contains potent agglutinins, the infant is perfectly normal though its red cells contain the specific agglutinogen. These puzzles have been solved by the discovery[1,2,3] that individuals sensitized to agglutinogens such as Rh, Hr, A, B, etc., may form 2 sorts of antibodies, namely, agglutinins (bivalent antibodies) and/or blocking antibodies or glutinins (univalent antibodies). The clinical manifestations depend on the type of isoantibody present in the maternal serum, so according to the present writer's theory erythroblastosis fetalis comprises 2 disease syndromes instead of only one. The characteristics of these disease syndromes and the author's proposed terminology are as follows:

(1) *Congenital hemolytic disease.* This syndrome is caused by the presence in the maternal serum of univalent antibodies specific for the fetal red cells. When only small amounts of antibody get into the fetal circulation, the infant is born alive, but develops an anemia which readily responds to proper transfusion therapy. When larger

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* Aided by a grant from the United Hospital Fund of New York City.

[1] Wiener, A. S., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 173.

[2] Wiener, A. S., *J. Lab. and Clin. Med.,* 1945, **30**, 662.

[3] Wiener, A. S., *J. Lab. and Clin. Med.,* 1945, **30**, 957.

amounts are present in the maternal serum, so that more passes into the fetus, the process progresses further *in utero* until the degree of anemia is such that anoxemia of the capillary walls permits exudation of serum proteins into the tissue spaces. A stillbirth results, or a living infant with hydrops, an almost invariably fatal condition. In these "pale" cases the characteristic pathological findings are marked pallor, ascites, anasarca, splenomegaly, hepatomegaly, and microscopically extramedullary islands of hematopoiesis in the liver and spleen and hemosiderosis of the liver and spleen.

(2) *Erythroblastosis proper.* Since agglutinins are presumably larger molecules than blocking antibodies, these only rarely traverse the placenta during pregnancy. During labor and delivery, however, increased intrauterine pressure may milk agglutinins into the fetal circulation. In such cases, physical examination of the newborn infant reveals no abnormality at first, but within a few hours a deep jaundice, with little or no anemia supervenes; the infant takes its feedings poorly, becomes stuporous, may have convulsions; coma and death usually supervene. The syndrome is due to the agglutination of the fetal red cells with the formation of agglutination thrombi in the smaller vessel and resulting damage to the liver and brain, in particular. Postmortem examination of such "toxic" cases shows generalized icterus, splenomegaly, and hepatomegaly; microscopically agglutination thrombi in the vessels of the brain are readily demonstrable. Thus, kernicterus is not caused by the jaundice *per se,* but represents an *in vivo* staining reaction of ganglion cells damaged by interference with the circulation. Fortunately, only few infants with this syndrome survive. Those that do are susceptible to the development of signs of a severe neurological disorder, characterized particularly by choreoathetosis, often associated with mental deficiency, and cirrhosis of the liver.

The new concept of 2 distinct clinical entitites instead of one has important implications in the prognosis, treatment and prophylaxis of the 2 disorders. It is now evident that the statement that modern transfusion therapy may only serve to save infants for the worse fate of mental deficiency is misleading, because in the author's experience all of 20 infants saved by such treatment have developed into perfectly normal children. Fortunately, the infants with the syndrome of erythroblastosis who are apt to develop mental deficiency as a sequel rarely survive.

15330

Antibiotic Activity of Extract of Western Red Cedar Heartwood.

CHESTER M. SOUTHAM.* (Introduced by B. C. Seegal.)

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It is common knowledge that certain woods are highly resistant to decay. One such wood is western red cedar (*Thuja plicata* D. Don) which when extracted in hot water yields a substance antibiotic to wood-destroying fungi.[1,2,3] The present paper is a study of the activity of this material against microorganisms pathogenic to man.

The hot water extract of western red cedar

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* The author wishes to thank the many people of the Columbia University College of Physicians and Surgeons who by encouragement and ready cooperation have made this study possible. He is especially indebted to Dr. E. T. Engle of the Department of Anatomy.

[1] Schmitz, H., *Idaho Forester,* 1922, **4**, 46.

[2] Sowder, A. M., *Ind. and Eng. Chem.,* 1929, **21,** 981.

[3] Southam, C. M., and Ehrlich, J., *Phytopath.,* 1942, **33**, 517.

TABLE I.
Growth of Various Bacteria on Buffered Nutrient Agar Containing Western Red Cedar Extract.*

Organism	Concentration of WRC extract in medium		
	57 mg%	115 mg%	162 mg%
Streptococcus hemolyticus (15A)	0	0	0
’’ ’’ (C203)	0	0	0
’’ *viridans*	+++	++	++
’’ *fecalis*	+++	++	++
Staphylococcus aureus (Oxford)	+++	+	0
’’ ’’ (B.D.)	++	++	++
’’ *albus*	+	0	0
Bacillus subtilis	++	++	0
Corynebacterium xerose	0	0	0
Mycobacterium tuberculosis (H37)	++++	++++	+
’’ *smegmatis*	++++	++++	not tested
Klebsiella pneumoniae	++++	+++	++
Eberthella typhosa	+++	++	+
Salmonella schottmülleri	+++	++	++
’’ *paratyphosa*	++++	+++	++
Shigella dysenteriae	0	0	0
’’ *sonnei*	0	0	0
’’ *paradysenteriae*	0	0	0
Escherichia coli (comunis)	++++	++++	+++
’’ ’’ (hemolyticus)	++++	+++	+++
Alcaligenes fecalis	0	0	0
Proteus vulgaris	++	++	+
’’ ’’ (OX19)	++	++	+
Pseudomonas aeruginosa	++++	++++	+++

* ++++ indicates growth equal to that on control plates.
+++ ’’ ’’ distinctly but not markedly less than on control plates.
++ ’’ ’’ markedly inhibited.
+ ’’ ’’ of a few colonies only.
0 ’’ no growth.

heartwood (hereafter designated WRC extract) was prepared by covering freshly made cedar sawdust[†] with distilled water, and simmering for 48 hours. The crude filtrate from this decoction was used in all tests. Concentration is expressed as mg of total solids per 100 cc of solution (mg%). This method is inaccurate because there are probably inert compounds in the extract, and because batches of extract from different trees have shown as much as fourfold differences in antibiotic activity in a given concentration.[‡] Most figures reported here were obtained with a single batch of extract made in 1945. In some of the studies with bacteria another, less effective, batch of extract was used, but such data have been recorded as those concentrations of the 1945 batch having an equal antibacterial activity.

The media used were prepared by making a stock beef infusion broth or agar twice as concentrated as usual, and containing disodium and monosodium phosphates to give a pH of 7.2 and a concentration of 0.04 molar phosphate. Just before use the desired amount of WRC and enough additional water to double the original volume of the medium was added. Buffering was necessary to eliminate antibiotic activity due solely to the acidity of the WRC extract. As prepared, the reaction of the media varied from pH 7.2 with low concentrations of extract to a maximum acidity of pH 6.6 with the highest concentrations of extract. The solid media were streaked with 24-hour broth cultures

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[†] Wood supplied by B. J. Carney & Co., Spokane, Washington.

[‡] WRC extract concentrations have been reported in terms of g of *wood* represented per 100 cc of extract by Southam and Ehrlich.[3] A "10%" concentration, as used in that publication, had approximately the same antibiotic activity as a "100 mg%" concentration in the present study.

TABLE II.
Growth of Fungi in Media Containing WRC Extract. (Symbols explained in Table I.)

		Concentration of WRC extract in media							
		Buffered medium				Unbuffered medium			
Fungus	Culture No.	57 mg%	115 mg%	162 mg%	345 mg%	15 mg%	29 mg%	57 mg%	115 mg%
Cryptococcus neoformans	1473	+	0	0	0	+	+	0	0
” ”	3	+++	++	+	+	+++	+++	0	0
Candida albicans	21	++++	++	+	0	++++	++++	+++	0
” ”	22	+++	++	++	0	++++	++++	+++	0
” ”	23	++++	+++	+	0	++++	++++	+++	0
” ”	24	++++	+++	+	0	++++	++++	++	0
Trichophyton gypseum	25	++	++	0	0	+++	++	0	0
” ”	26	+	+	0	0	+++	+++	0	0
” *purpureum*	27	++	+	0	0	+++	++	++	0
” ”	28	++	+	0	0	+++	++	0	0
” ”	29	++	+	0	0	++++	++++	0	0
Epidermophyton floccosum	30	+	+	0	0	++++	++++	+	0
Microsporum felineum	31	++	++	0	0	+++	++	0	0
” *audouini*	33	++	+	+	0	+++	++	0	0
” ”	34	++	0	0	0	+++	++	+	0
” ”	35	+	0	0	0	+	+	0	0

of the test organism. Liquid media were inoculated with 0.1 cc of an undiluted 12-hour broth culture of the test organism. Degree of growth after 24 hours incubation at 37°C on WRC containing media was estimated by comparison with growth on control medium. Growth was recorded as normal (++++), slight stasis (+++), marked stasis (++), very slight growth (+), and no growth (0). *Myc. tuberculosis* and *Myc. smegmatis* were grown in test tubes on glycerol-agar containing WRC extract and were incubated for 8 weeks, and 4 days, respectively. Santon's synthetic liquid medium containing WRC extract was inoculated with bits of 3-week-old pellicle of *Myc. tuberculosis* and incubated 4 weeks.§

Degrees of growth of a variety of bacteria on agar media in the 3 concentrations of WRC extract tested are recorded in Table I. It will be seen that there is no relation between staining reaction or morphology of the organisms and their susceptibility to the antibiotic action of WRC extract. There is great variation in susceptibility among different species. Results similar to those recorded were obtained by using liquid medium plus WRC extract.

Fungi pathogenic to man were studied by similar technic, with both buffered and unbuffered malt agar medium (2½% desiccated malt extract in 2% agar). The yeast-like fungi (*Cryptococcus* and *Candida*) were streaked in the same manner as bacteria from 4-day maltose broth cultures and the results were read after 4 days. Filamentous fungi were studied by making a single planting 5 mm in diameter in each concentration and comparing the rate of peripheral growth of the mycelium with that on control medium after 7 days. Degree of growth is recorded in the same way as for the bacteria. Incubation was at 37°C.

Results of the fungus growth studies are summarized in Table II. In general the tested fungi were inhibited by the same range of concentrations of WRC extract as the bacteria, when on buffered medium, and by ¼ to ½ these concentrations on unbuffered medium. Fungi causing wood decay are inhibited by similar concentrations.[3]

The effect of WRC extract on the growth of the Shiga dysentery bacillus was studied by the colony-count (plating-out) technic. Before incubation there were 10,000 viable organisms per cc of medium (buffered nutrient broth plus WRC extract) in all tubes. The control tubes contained 10,000,000 viable

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§ Tests using Santon's medium were conducted by C. J. Duca, Department of Bacteriology.

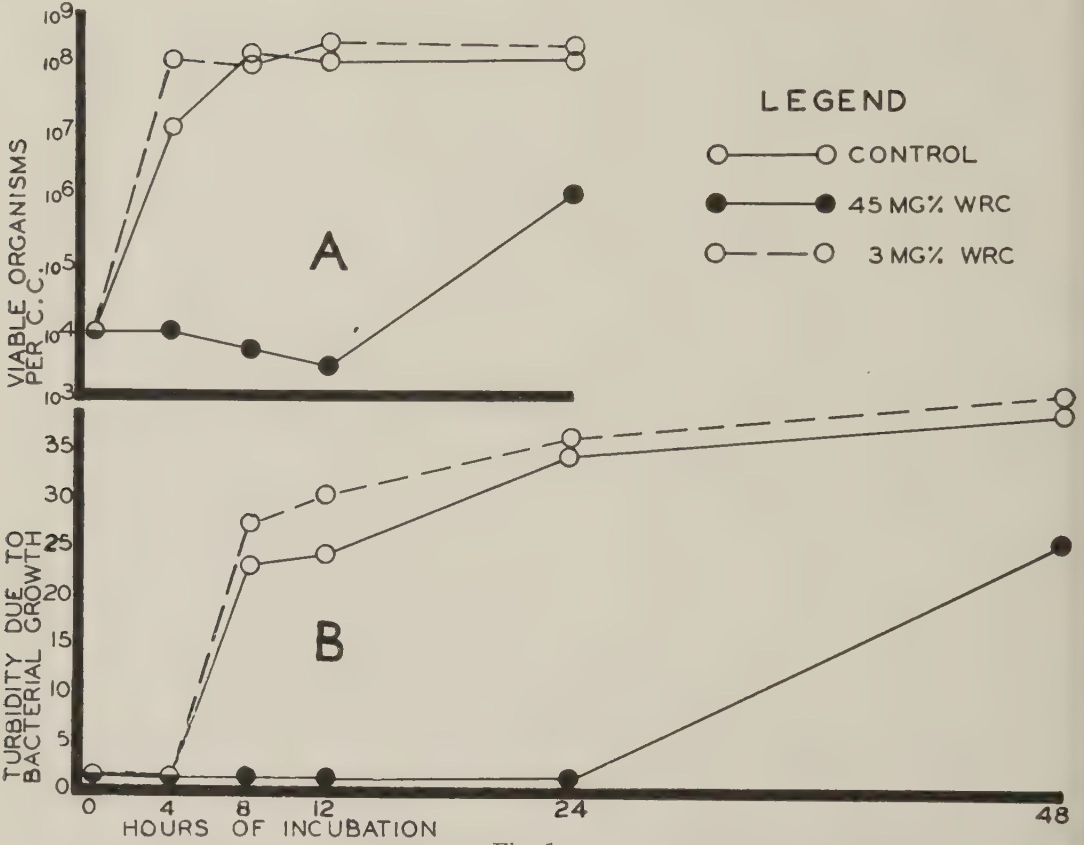

Fig. 1.

Growth of *Shigella dysenteriae* in buffered nutrient broth containing WRC extract and in control medium. (a) Colony-count technic; (b) turbidimetric technic.

organisms per cc after 8 hours incubation at 37°C. There was no significant change in number of viable organisms in the next 16 hours. In the tubes containing 45 mg% WRC extract there was no increase in viable organisms during the first 12 hours of incubation, but there was an increase to 1,000,000 after 24 hours. These results show that the anti-bacterial activity of WRC extract in the concentration tested is due to stasis, not to death, of the organisms (Fig. 1A). A second experiment by this same technic confirmed these results.

Growth was also studied in these same tubes by turbidimetric readings taken simultaneously with sampling for the colony-count studies. A Klett-Summerson photo-electric colorimeter was used. A dark red (No. 630) filter eliminated variations due to differences in depth of color of the media. Readings were obtained in units of optical density (log incident light/log transmitted light). Each reading designated in Fig. 1B was obtained by subtracting the reading obtained before incubation from that obtained after the indicated period of incubation. These figures therefore represent turbidity due to bacterial growth, not total turbidity of the culture.|| These results (Fig. 1B) also demonstrate that WRC extract is bacteriostatic rather than bactericidal. These results agree with previous findings as to mode of action of WRC

|| Comparison of the growth curves obtained simultaneously by the 2 technics (Fig. 1A and 1B) shows that the cultures contained nearly the maximum number of *living* organisms before any turbidity could be detected (*i.e.* after 4 hours incubation in the controls). Increase in turbidity after this time must be due to accumulation of dead cells.

against wood-destroying fungi.[3]

Microscopic examination of suppressed cultures of several species revealed many elongated rods or enlarged cocci, as has been described previously for organisms exposed to other antibiotics.[4]

The mechanism of the bacteriostatic action of WRC extract is unknown. However, it was learned that at room temperature at pH 7.4 WRC extract reacts instantaneously with cysteine in evacuated Thunberg tubes in such manner that the cysteine is rendered unable to reduce methylene blue. This reaction required a minimum of approximately 230 mg of WRC extract solids per milequivalent weight of cysteine hydrochloride (½ cc of 1:5000 methylene blue as indicator). WRC extract does not act as a general oxidizing agent for other easily oxidized substances (KI H_2S, Fe ++) so it is probable that the WRC extract does not oxidize cysteine to cystine, but rather reacts directly with the sulfhydryl group. If this be true, it is possible that reaction of WRC extract with sulfhydryl compounds is the mechanism, or one of the mechanisms, of its antibiotic action.[5] The product of reaction of WRC extract with cysteine has no antibiotic activity. WRC extract is also inactivated by human serum and blood. It is not affected by normal urine or by gastric residue.

The dose of WRC extract lethal for animals has not been determined. Mice were maintained for 4 weeks drinking no liquid other than WRC extract (230 mg%), ingesting approximately 230 mg of solids per mouse (11.5 g/K), without toxic symptoms, without weight loss, and without change in volume of liquid consumed. A single subcutaneous injection of 140 mg of WRC extract solids into mice had no ill effects and appeared to cause no discomfort. Intravenous doses of 230 mg (50 cc volume) twice a day for 3 days in a rabbit, making a total of 3 g/K, caused no apparent ill effects, and caused no grossly detectable pathology. Six mice which were each injected intraperitoneally with 7 mg doses twice a day for 6 days (total of 4.3 g/K) showed no toxic manifestations. These mice were also drinking WRC extract (230 mg%) throughout this period as their sole source of liquid. Autopsy of 3 of these mice revealed a few scattered discrete white areas up to 2 mm in diameter in their livers. Histological examination revealed that these were fibrosing areas of necrosis containing polymorphonuclear cells as well as macrophages. No other gross pathology could be detected. Kidneys were histologically normal. No other tissues were sectioned. This hepatic pathology may be an effect of the WRC extract. However, the absence of similar pathology in the rabbit, and the discrete character of the lesions, suggests that this pathology may have had some other, possibly infectious, etiology.

Limited trials have failed to reveal any significant antibiotic effect of WRC extract against systemic infection in mice by pneumococcus type III, *Streptococcus hemolyticus* strain C203, *Candida albicans*, or *Cryptococcus hominis*. The possibility of its use against skin infections has not yet been investigated.

The bacterial content of the cecums of normal mice and of mice which had been drinking WRC extract as their only liquid for the preceding week was studied by emulsifying 1 mg of the cecal contents in 10 cc of broth, diluting serially, and growing out in nutrient agar. No significant reduction in number of bacteria was caused by the WRC extract.

Urine from mice and rabbits injected with WRC extract was found to have a greater antibacterial effect than urine from control animals. It seems probable therefore, although the results are not conclusive, that WRC extract is excreted in part in bacteriostatic form by the kidneys.

WRC extract is completely dialyzable through cellophane membrane, but does not diffuse through agar. Boiling to dryness causes little or no loss of antibiotic activity. Heating to 200°C (in sealed vials in boiling benzyl alcohol) momentarily or for 10 minutes resulted in loss of 30% of activity. Acidification to pH 1 with HCl, for 48 hours, followed by neutralization with NaOH, caused no detected loss of activity. Alkalinization to pH 12 for one hour, followed by neutralization with HCl, caused no detected loss of activity, but when held at this pH for 48 hours there was roughly 50% loss of activity. At least 10% of the antibiotic activity resides in ether-soluble resinous components of the extract. The active components can be almost completely removed from WRC extract by extraction with benzyl alcohol or by adsorption onto norite, but the active components have not yet been recovered from these reagents.

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[4] Waksman, S. A., Microbial Antagonisms and Antibiotic Substances, Commonwealth Fund, 1945, **50**, 213-215.

[5] Cavallito, C. J., Bailey, J. H., Haskell, T. H., McCormick, J. R., and Warner, W. F., *J. Bact.*, 1945, **50**, 61.

Summary. Water extract of western red cedar heartwood inhibits growth of a wide variety of bacteria and fungi. Inhibition is due to stasis, not to death, of the organisms. Susceptibility of microorganisms is unrelated to Gram-staining reaction or to morphology.

WRC extract does not lose its antibiotic activity when subjected to prolonged boiling, or considerable changes in pH. It is not inactivated by urine or gastric residue, but is inactivated by blood, serum, and cysteine.

Large doses of the extract (4.3 g/K I.P. in mice, 3 g/K I.V. in a rabbit) did not cause death or illness.

No *in vivo* activity against infection has yet been demonstrated.

15331

Immunological Studies of Dengue Fever and Colorado Tick Fever.

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(Introduced by Max Levine.)

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Ashburn and Craig[1] demonstrated, by human inoculation studies, that dengue fever was caused by a filterable agent and that it was transmitted by mosquitoes. By similar means it has been demonstrated that Colorado Tick fever is caused by a filterable agent,[2] but it appears that the development of natural cases of the disease is associated with tick bites.[3] The Colorado tick fever and the dengue fever syndromes are both characterized by generalized malaise, headache, leucopenia, and a frequent "saddle-back" or di-phasic temperature curve. They are both self-limiting and non-fatal. The outstanding differential feature seems to be the development of a skin rash with many cases of dengue fever and no rash with Colorado tick fever; also an association with ticks in the case of Colorado tick fever and with mosquitoes in the case of dengue fever. In view of the similarity of the clinical pictures of these 2 diseases and since they are both associated with arthropods, some opinions have been expressed[4] that they both may be caused by related agents.

Human Experiments. Since animals are not highly susceptible to either of these agents, it was considered advisable to study their immunological relationship to each other by human inoculation experiments.

Two normal male volunteers were selected for subcutaneous inoculation with 1.0 cc of the Colorado Tick fever agent* (Case I-A.B.

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[1] Ashburn, P. M., and Craig, C. F., *Philippine J. of Science*, 1907, B, **2**, 93.

[2] Florio, Lloyd, Stewart, M. O., and Mugrage, E. R., The Etiology of Colorado Tick Fever, *J. Exp. Med.*, 1946, **83**, 1.

[3] Topping, Norman H., Cullyford, J. S., and Davis, G. E., *Public Health Rep.*, 1940, **55**, 48.

[4] Rogers, L., and Megaw, J. W. D., *Tropical Medicine*, The Williams and Wilkins Co., 1944, 146.

* We wish to express our sincere appreciation to Dr. E. R. Mugrage, University of Colorado

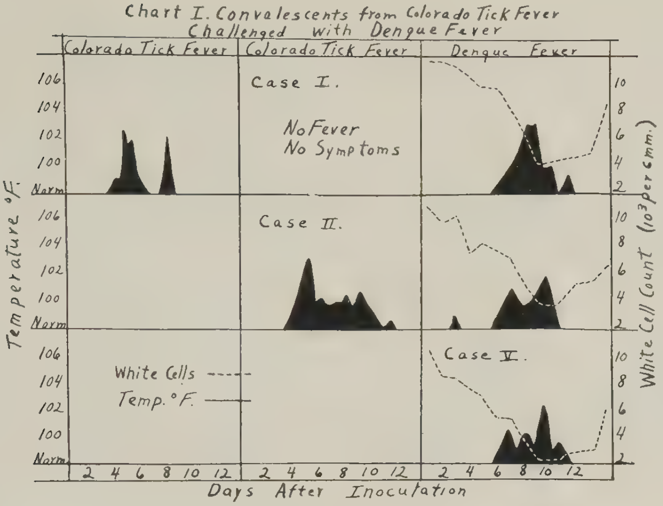

Fig. 1.
Case I inoculated with Colorado Tick Fever, challenged first with Colorado Tick Fever, then with Dengue Fever. Case II inoculated with Colorado Tick Fever, then challenged with Dengue Fever. Case V inoculated as control for Dengue Fever Virus at time of challenging Cases I and II.

and Case II-A.P.R.) As noted in Fig. 1, Case I had manifested experimentally induced Colorado tick fever and was immune when challenged 5 months later with the same strain of the disease agent. The control inoculation (Case II) developed the disease after 4 days and remained ill for 8 days. The inoculum had been collected on the first day of fever from an experimentally induced case of Colorado tick fever and had thereafter been stored for 6 months in the dry ice chamber. Blood serum was collected from Case II on the first day of fever and was stored in the dry ice chamber for future use.

Twenty-seven days after defervescence, the 2 Colorado tick fever convalescent patients and one normal control were inoculated intradermally with 0.4 cc of dengue fever infected serum. The inoculum was collected from Case IV which is described below. All 3 of the volunteers developed a syndrome which was considered compatible with the clinical diagnosis of dengue fever: fever, leucopenia, generalized malaise and rash.

Two normal male volunteers (Case III-C.W. and Case IV-E.T., Fig. 2) were inoculated intradermally with 0.4 cc of dengue fever infected serum (second human passage).§ Six days after this inoculation the patients developed symptoms of the disease and the illness persisted for 6 days. Blood serum was collected from these patients during the first day of illness and was stored in the dry ice chamber for future use. Twenty-seven days after defervescence the 2 dengue fever convalescent patients and one normal control were inoculated subcutaneously with 1.0 cc of Colorado tick fever infected serum. This inoculum had been collected from Case II described above. Three and 4 days after the inoculation all of the challenged individuals and the control developed the syndrome of Colorado tick fever (Fig. 2): fever, leucopenia, generalized malaise, but no rash.

This reciprocal challenge experiment, therefore, failed to demonstrate any relationship between the dengue fever and the Colorado tick fever strains which were employed.

Discussion. There are several diseases reported in the literature, all of which are characterized by fever, by generalized malaise, by leucopenia and by being associated with arthropod infestation. Speculations have been

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Medical School, for supplying us with Colorado tick fever infected hamster serum.

§ The dengue fever agent was kindly supplied by Dr. A. B. Sabin, Children's Hospital Foundation, the University of Cincinnati.

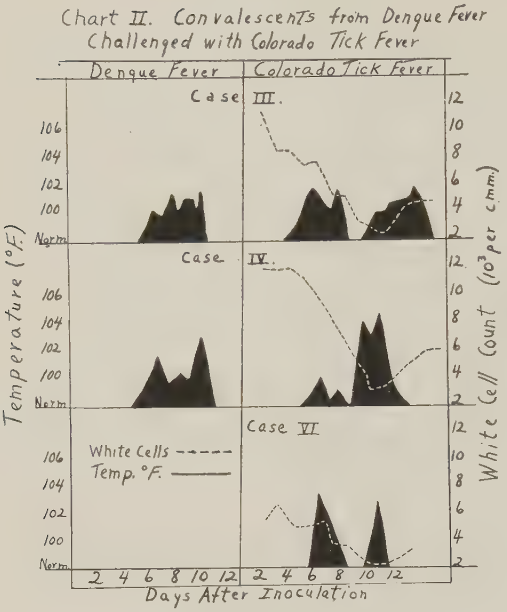

Fig. 2.
Cases II and IV inoculated with Dengue Fever then challenged with Colorado Tick Fever. Case VI inoculated with Colorado Tick Fever for control.

proposed concerning their possible similarity in etiology. To great extent, experimental results have served to delineate their relationship to each other. Sandfly fever and dengue fever appear to be caused by immunologically distinct agents.[5] Bullis fever and Colorado tick fever, while both associated with tick bites, appear to be of different etiology;[6] furthermore, Bullis fever and dengue fever have not thus far demonstrated any immunological relationship.[7]

Different strains of the same disease may vary somewhat immunologically. It is possible that the relationship of dengue fever and Colorado tick fever with strains other than those employed herein, may not coincide with the results reported. It appears, from our results, however, that there is no immunological relationship between the agents of Colorado tick fever and of dengue fever (strain).

Summary. On the basis of the experimental data presented, there does not appear to be any immunological relationship between dengue fever and Colorado tick fever.[†]

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[5] Sabin, A. B., Philip, C. B., and Paul, J. R., *J. Am. Med. Assn.*, 1944, **125**.

[6] Pollard, M., Livesay, H. R., Wilson, D. J., and Woodland, J. C., Experimental Studies with Bullis Fever, *Am. J. Trop. Med.*, 1946, in press.

[7] In press.

[†] A recent article by Florio, Hammon, Laurent, and Stewart appeared in the *Journal of Experimental Medicine*, 1946, **4**, 83, in which essentially the same results were obtained as is reported in this paper.

Grateful acknowledgment is made to Major Choice Matthews, M. C., and Captain Sidney Cohen, M. C., for assistance with the clinical observations.

15332

A Hemorrhagic Syndrome Induced by Derivatives of 3-Hydroxy-1, 4-Naphthoquinone.*

CARL C. SMITH, ROCHELLE FRADKIN, AND MARY D. LACKEY.
(Introduced by L. H. Schmidt.)
From the Institute of Medical Research, The Christ Hospital, Cincinnati, Ohio.

During the routine examination of the short-term chronic toxicities of various antimalarial drugs for the rat, it was noted that certain derivatives of 3-hydroxy-1,4-naphthoquinone produced a fatal hemorrhagic syndrome associated with a profound hypoprothrombinemia. The production of such a syndrome by drugs related closely in structure to the antihemorrhagic K vitamins, the rapidity with which this syndrome developed and its severity were all items of interest. Especially significant, however, was the finding that production of hemorrhagic lesions and hypoprothrombinemia by at least one of the above naphthoquinones was blocked by simultaneous administration of vitamin K_1. A description of these observations is presented here.

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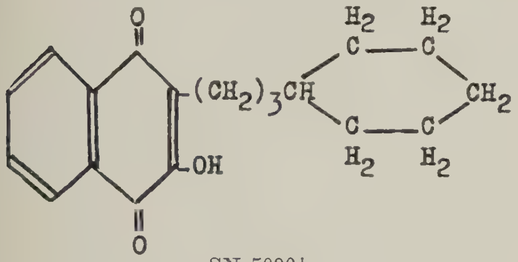
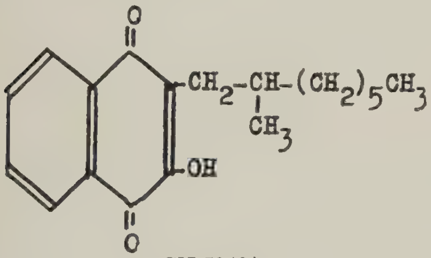
* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Institute of Medical Research, The Christ Hospital, Cincinnati, Ohio.

Experimental. The compounds studied were the following:



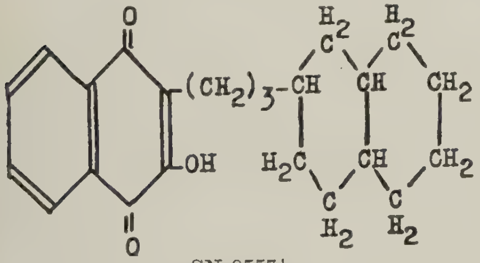
SN 5090†

2-(3-cyclohexyl-propyl) - 3 - hydroxy-1,4-naphthoquinone.



SN 5949†

2-(2-methyloctyl)-3-hydroxy-1,4-naphthoquinone.



SN 8557†

2-[3-(decahydro-2-naphthyl)propyl]-3-hydroxy-1, 4-naphthoquinone. (Trans mixture).

The toxicities of these substances were studied in the young rat in accordance with the standardized technics employed with other antimalarial drugs. Since these procedures will be described in detail elsewhere‡ only the most important features will be mentioned here. The drugs, suspended either in water or in olive oil, were administered at various dose levels to groups of young rats for periods of 8 to 11 days. Treatments were given once daily via stomach tube. Control rats received similar quantities of water or olive oil without drug at the same intervals. The weights of the animals were obtained daily. In many instances, prothrombin times were measured. Samples of whole blood obtained by cardiac puncture were used for this purpose. Determinations were carried out according to the method of Kato[1] using Russell viper venom[2] as the source of thromboplastin.

Twenty-eight-day-old male rats, Sprague-Dawley strain, were used throughout the study. The weights of these animals at the start of an experiment averaged 55 g. Throughout the treatment period the rats were fed Purina Dog Chow Checkers and water ad libitum.

A brief summary of the pertinent results of the study is given in Table I. The data presented there show that at certain dose levels each of the 3 compounds produced a hemorrhagic syndrome in the young rat. The first evidence of this syndrome usually appeared after the third or fourth day of treatment. At this time the ears, feet and tails showed unusual pallor and in some cases there was slow bleeding from ear puncture wounds made to identify the animals. Body temperatures were reduced and the rats were weak and inactive. Twenty-four to 72 hours later the animals were usually moribund. Gross extravasations of blood into the subcutaneous tissues of the legs, neck and head, and about the testicles were invariably present. Blood samples obtained from the heart without anticoagulant either failed to clot within 24 hours or clotting was greatly delayed. The hematocrits on such samples

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† The numbers cited here are those assigned to the compounds by the Survey of Antimalarial Drugs, Baltimore, Maryland. The drugs were prepared by Dr. Louis F. Fieser, Harvard University, and the Abbott Laboratories, to whom we are greatly indebted.

‡ *Survey of Antimalarial Drugs, 1941-1945,* to be published in July, 1946.

[1] Kato, K., *Am. J. Clin. Path.*, 1940, **10**, 147.

[2] Page, R. C., and Russell, H. K., *J. Lab. and Clin. Med.*, 1941, **26**, 1366.

TABLE I.
Observations on the Toxicities of Derivatives of 3-Hydroxy-1,4-Naphthoquinone.

Daily dose mg/kg	No. of rats treated	Deaths due to treatment	Wt change in % controls*	Effects of treatment
				SN 5090 — administered in water
400	6	0	130	No manifestation of toxicity during 8 days treatment.
800	6	0	130	
1600	6	1	75	Hemorrhagic syndrome appeared in 3 rats on 5th and 6th days.
				SN 5090 — administered in olive oil
800	4	1	12	Greatly prolonged prothrombin times in 3 rats surviving 11 days treatment.
1200	3	3	—	Fatal hemorrhagic syndrome developed in all 5 animals; mean survival time 5 days.
1600	2	2	—	
				SN 5949 — administered in water
100	6	0	140	No manifestation of toxicity in treatment period of 8 days.
200	6	0	85	
400	6	2	75	Hemorrhagic syndrome appeared in 2 rats in 5 days; mean survival time 5 days.
				SN 5949 — administered in olive oil
100	5	0	92	No manifestation of toxicity during 11 days treatment.
200	5	0	84	
400	5	5	—	Fatal hemorrhagic syndrome developed in all 5 rats; mean survival time 4 days.
				SN 8557 — administered in water
100	6	0	93	No manifestation of toxicity during 11 days treatment.
200	6	4	—	Fatal hemorrhagic syndrome in 4 animals; mean survival time 5 days.
400	6	6	—	Fatal hemorrhagic syndrome in all 6 rats; mean survival time 6 days.
				SN 8557 — administered in olive oil
100	6	0	57	No manifestation of toxicity in treatment period of 11 days.
200	6	5	—	Fatal hemorrhagic syndrome in 5 animals; mean survival time 5 days.
400	6	6	—	Fatal hemorrhagic syndrome developed in all 6 rats; mean survival time 6 days.

* The figures in this column were obtained as follows:

$$\frac{\text{G wt change of treated rats}}{\text{G wt change of control rats}} \times 100$$

were extremely low—in some instances only 15%. Necropsies presented a fairly uniform gross picture—bloodless appearance of such organs as liver, kidney, spleen and pancreas; gross hemorrhage into the abdominal cavity and the upper portions of the gastro-intestinal tract without distinct ulceration; considerable partially digested blood in the lower parts of the digestive tract.

The first appearance of this syndrome suggested the presence of a prothrombin deficiency. Consequently prothrombin times were measured routinely whenever blood samples could be obtained. The results of these determinations may be summarized briefly. In most instances prothrombin times were normal in animals which showed no bleeding tendency; in a very few cases, however, there were slight but significant elevations of the prothrombin times. In contrast to this, rats presenting the hemorrhagic syndrome had greatly prolonged prothrombin times, varying from 300 seconds to in excess of 24 hours. With the method employed here, normal rats have a prothrombin time of 18 ± 3 seconds.

It should be pointed out that there were some quantitative differences in the toxicities of the 3 drugs employed here. SN 5090 was distinctly less toxic than SN 5949 or SN 8557. SN 5090 appeared to be considerably more toxic when administered as a suspension in olive oil than when suspended in water. The menstruum had little effect on the toxicities of SN 5949 and SN 8557.

In both the experiments described above and in collateral studies, data have been sought which would explain the observed hypoprothrombinemia. One of the first possibilities considered was that the hypoprothrombinemia resulted from hepatic injury. This explanation does not seem likely, for in only one of the rats was there distinct microscopic evidence of liver pathology. In the liver of this animal there was marked fatty degeneration and areas of coagulation necrosis about the central vein. It is noteworthy that more severe changes than these have been observed in hundreds of rats receiving other potential antimalarial drugs, but in none of these animals was there a hemorrhagic syndrome or evidence of hypoprothrombinemia.

The second explanation which suggested itself was that, as in the case of the indandiones,[3] another group of hemorrhagic agents, the naphthoquinones effected a direct inactivation of prothrombin. To determine this point, prothrombin times were measured before and at various intervals up to 8 hours after normal rat plasma had been saturated with either SN 5090 or SN 5949. Without exception prothrombin times were the same in plasmas saturated with these drugs as in the control plasmas. This result argues against direct inactivation of prothrombin by the naphthoquinones.

Another possible explanation was that the hypoprothrombinemia resulted from a vitamin K deficiency induced by interference with bacterial synthesis of these vitamins in the intestine—an effect not unlike that induced by the sulfonamides.[4] *In vitro* experiments have shown, however, that neither SN 5090 nor SN 5949 possesses significant bacteriostatic or bactericidal activities against *Escherichia coli*,[5] the intestinal organism regarded as the principal bacterial source of the K vitamins.[6] Furthermore, the hypoprothrombinemia associated with sulfonamide therapy develops only after weeks or months of treatment; the hypoprothrombinemia described here appears in a matter of a few days. Thus, at the present moment there seems little to support the explanation suggested above.

Another explanation was that drugs such as SN 5090 competed with the K vitamins in the formation of prothrombin—the relationship being not unlike that which appears to exist between the sulfonamides and p-aminobenzoic acid, or between pantoyltaurine and pantothenic acid. The explanation suggested itself, not only because of the structural similarities of substances with vitamin K activity and the above hemorrhagic agents, but also because of Richardson's finding,[7] that the activities of the above naphthoquinones against infections with *Plasmodium lophurae* were at least partially blocked by 2-methyl-1,4-naphthoquinone, sometimes called synthetic vitamin K.

To test the above postulate, an experiment was set up to determine whether the toxic reactions produced by SN 5090 could be blocked by simultaneous administration of either vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) or 2-methyl-1,4-naphthoquinone. The results of this experiment, summarized in Table II, show rather dramatically that the simultaneous administration of vitamin K_1 at a daily dosage level of 10 mg per kg blocked completely the appearance of the hemorrhagic syndrome and hypoprothrombinemia in rats receiving SN 5090. Furthermore, vitamin K_1 blocked the

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[3] Kabat, H., Stohlman, E. F., and Smith, M. I., *J. Pharm. and Exp. Therap.*, 1944, **80**, 160.

[4] Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P., *J. Biol. Chem.*, 1942, **145**, 137.

[5] Sesler, C. L., and Schmidt, L. H., unpublished observations.

[6] Dam, H., *Advances in Enzymology*, 1942, **2**, 285.

[7] Personal communication from L. F. Fieser.

TABLE II.
The Effects of 2-Methyl-1,4-Naphthoquinone and Vitamin K_1 on the Toxicity of SN 5090.*

Rat No.	Days of treat-ment	Wt, g Initial	Wt, g Final	Prothrombin time† sec.	Remarks
Rats received 800 mg SN 5090 per kg body weight, daily					
1	4	57	53	240	Moribund, sacrificed on 5th day; fatal hemorrhagic lesions.
2	4	56	54	180	
3	7	54	57	—	Died 7th day; fatal hemorrhagic lesions.
4	7	58	70	—	" 8th " " " "
5	8	55	67	180	Moribund, sacrificed 8th day; fatal hemorrhagic lesions.
6	8	57	70	240	
Rats received 800 mg SN 5090 and 10 mg 2-methyl-1,4-naphthoquinone per kg body weight, daily					
7	12	56	70	150	Sacrificed end of treatment; moderately severe hemorrhagic lesions.
8	12	64	88	150	
9	12	57	88	95	
10	12	60	101	95	
11	12	50	90	50	Sacrificed end of treatment; nothing remarkable.
12	12	59	100	40	
Rats received 800 mg SN 5090 and 10 mg vitamin K_1 per kg body weight, daily					
13	12	51	93	20	Sacrificed end of treatment; nothing remarkable.
14	12	60	117	19	
15	12	56	108	17	
16	12	55	96	15	
17	12	58	100	15	
18	12	65	112	15	

* SN 5090 was suspended in olive oil; the vitamins were dissolved in olive oil.

† Prothrombin times were obtained when animals were sacrificed, either moribund or at end of experiment.

growth inhibiting effects of SN 5090; animals receiving both of these naphthoquinones (rats 13-18) gained weight essentially as well as olive oil controls. Although methylnaphthoquinone was much less effective in counteracting the toxicity of SN 5090 than vitamin K_1, it did delay the appearance of the hypoprothrombinemia significantly, retarded the development of hemorrhagic lesions and partially blocked the growth inhibiting effects of SN 5090. These data would seem to support the postulate that naphthoquinones like SN 5090 produce a hypoprothrombinemia by competing with the K vitamins in processes essential to prothrombin formation.

Discussion. The results of the present study have shown that 2-substituted-3-hydroxy-1,4-naphthoquinones, such as SN 5090, produce a hemorrhagic syndrome when administered to the rat in large doses. A rational explanation for this hemorrhagic activity has been offered, namely, that these quinones function as antimetabolites in whatever processes the K vitamins serve in prothrombin formation. The observation that vitamin K_1 and to a lesser extent methylnaphthoquinone antagonize the hemorrhagic and other toxic properties of SN 5090 provides good support for this explanation.

A number of other 3-hydroxy-1,4-naphthoquinone derivatives have been reported in the literature. It is significant that at least one of these derivatives, namely, 2,2′-methylene-bis-3-hydroxy-1,4-naphthoquinone, possessed hemorrhagic activity. This compound produced a significant reduction in plasma prothrombin content when administered to rabbits in doses of 20-40 mg.[8] The other 2-substituted-3-hydroxy-naphthoquinones were studied only for vitamin K activity in the chick at low dosage levels of not more than 1 mg.[9] Perhaps some of these compounds would have displayed hemorrhagic activity had they been tested in rats or rabbits in larger doses. Conversely, the 2-substituted-3-hydroxy-naphthoquinones used in the present study might display antihemorrhagic activities if assayed at low doses in the vitamin K-deficient chick.

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[8] Meunier, P., Mentzer, C., Buu-Hoï, and Cagniant, P., *Bull. soc. chim. biol.*, 1943, **25**, 384.

[9] Almquist, H. J., and Klose, A. A., *J. Am.*

Summary. 1. A fatal hemorrhagic syndrome accompanied by marked hypoprothrombinemia was produced in rats by the administration of 3 2-substituted-3-hydroxy-1,4-naphthoquinones.

2. These compounds produced only minimal pathologic changes in the liver, caused no measurable destruction of prothrombin *in vitro,* and possessed only slight bacteriostatic activity against *Escherichia coli,* the principal source of vitamin K in the gut.

3. The hemorrhagic and toxic properties of at least one of the compounds studied, SN 5090, were antagonized partially by 2-methyl-1,4-naphthoquinone and completely by vitamin K_1. On the basis of this finding it was postulated that these 2-substituted-3-hydroxy-1,4-naphthoquinones, such as SN 5090, compete with the K vitamins in processes essential to prothrombin formation and by displacing these vitamins produce the hemorrhagic syndrome described here.

Chem. Soc., 1939, **61**, 1924, 2557; Almquist, H. J., and Klose, A. A., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 55; Fieser, L. F., Bowen, D. M., Campbell, W. P., Fry, E. M., and Gates, M. D., Jr., *J. Am. Chem. Soc.*, 1939, **61**, 1926; Fieser, L. F., Campbell, W. P., and Fry, E. M., *J. Am. Chem. Soc.*, 1939, **61**, 2206; Fieser, L. F., Tishler, M., Sampson, W. L., and Woodford, S., *J. Biol. Chem.*, 1941, **137**, 659; Fieser, L. F., and Gates, M. D., Jr., *J. Am. Chem. Soc.*, 1941, **63**, 2948; Fernholz, E., and Ansbacher, S., *Science*, 1939, **90**, 215.

15333

Chemotherapy of Experimental Leishmaniasis.*

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The work here reported was an experimental chemotherapeutic investigation of a variety of organic compounds, both metallic and nonmetallic, to determine whether any offered a new approach to the treatment of visceral leishmaniasis. None as suitable as available drugs was discovered. This report will describe the method of evaluating drugs of unknown potency and summarize tests of 188 compounds.

Materials and Methods. Few details of the investigative technics of various authors have been published and, in most cases, these technics require much time and have not been well standardized. (A recently-described, accurate but time-consuming method is that of Goodwin).[1] A routine "screening" method should be reliable, rapid and convenient. The method to be described meets these qualifications. However, had a potent drug of low toxicity been discovered, the method of comparison with drugs already available would have had to be much more elaborate.

In all experiments the Syrian hamster, *Cricetulus auratus*, was used. It was clearly desirable that the dosage of a compound of unknown value be as high as infected hamsters could tolerate. Usually a satisfactory dose could be determined from the estimated murine LD50 following a single intraperitoneal injection of either an aqueous solution of a compound, or, if the compound was insoluble in water, a suspension in 2%

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

[1] Goodwin, L. G., *Trans. Roy. Soc. Trop. Med. Hyg.*, 1945, **39**, 133.

gum acacia. Ten per cent of the estimated murine LD50 was administered once daily intraperitoneally for 6 days to hamsters under treatment by the method outlined below. In some instances, serious toxic effects as judged by a fall in weight still occurred and adjustment of the daily dose to a lower level had to be instituted. The routinely used standard drug, the diethylamino ethanol of sodium antimony gluconate ("Stibanose" or "Solustibosan") had a very low toxicity represented by an LD50 and standard deviation, after a single intraperitoneal injection into mice, of 3550 ± 925 mg per kg. In contrast to this quinquevalent antimonial, a similar estimate of the LD50 and standard deviation of the tervalent antimonial, tartar emetic, was 60 ± 16 mg per kg.

Standardization of the method of testing routinely the chemotherapeutic action of drugs in experimental leishmaniasis required much time. Preliminary experiments with a strain of *Leishmania infantum,* kindly furnished by Dr. S. Adler of the Hebrew University in Jerusalem, produced infections after intrasplenic inoculation but was not satisfactory for routine use because of the difficulty of standardizing the infection. The same objection was found to hold when intratesticular inoculations were made with several strains of *Leishmania.* By using the Khartoum strain of *L. donovani,* which was obtained through Dr. Henry Meleney, and by making a rigidly controlled intraperitoneal inoculation of splenic emulsion, well standardized leishmaniasis in groups of hamsters could be produced.

In routine experiments, one or more groups of 100 hamsters about 4 weeks old received inoculation from a single suspension of ground hamster spleen which was heavily parasitized with the Khartoum strain of *L. donovani.* Each animal was given an intraperitoneal injection of uniformly mixed suspension equivalent to 20 mg of spleen. Careful precautions were taken to avoid bacterial contamination. After 4 weeks there was usually a patent infection which was uniform for the group as judged by liver smears obtained by puncture with a 22 gauge needle fitted to a syringe. Each group of 100 was subdivided at random into groups of 5 hamsters. Of these sub-groups, one served as an untreated control, a second received the standard drug, Stibanose, in a daily dose of 500 mg per kg, and the remaining 18 sub-groups received compounds of unknown value. All solutions or suspensions of compounds were injected intraperitoneally once daily for 6 days and a second liver puncture was made on the eighth day—2 days after the last injection. At this time, usually all the hamsters receiving Stibanose were free from leishmaniasis *as judged by liver smears;* however, in an occasional group, liver smears were still positive in 1 or 2 of the 5 animals. Two weeks later animals treated by Stibanose rarely had positive liver smears whereas cultures of liver in N.N.N. medium were positive in about 20%. The parasitization of control hamsters or of others receiving inactive compounds progressed rapidly until death owing to renal amyloidosis occurred.[2] Remarkably consistent evaluation of liver smears was obtained "blindly" by independent observers. Results could easily be classified as (1) no chemotherapeutic effect, (2) a chemotherapeutic effect less than that a Stibanose, and (3) a chemotherapeutic effect equal to that of Stibanose. Rarely, a difference between categories (1) and (2) could not be decided until further experiments had been completed. Additional experiments were performed with any drug falling in category (2) or (3).

Results. Tables summarizing data on all compounds not restricted with regard to publication can be secured from the authors. The greatest proportion of active drugs, as might be expected, was found among the 30 antimonials. Only 2 of the 16 tervalent antimony derivatives were active whereas 8 of the 14 quinquevalent antimonials were beneficial in experimental leishmaniasis. No new drug of these groups was as effective as known drugs such as Stibanose, Neostam, and Neostibosan. Fourteen other organometallic compounds containing arsenic, bismuth, copper, gold or mercury had no effect on the disease. None of 144 aliphatic, carbocyclic and heterocyclic non-metallic compounds was found to possess important antileishmanial effects. Only 4 of these 144 compounds, administered in large doses in terms of toxicity, had any effect; this effect was clearly inferior to that of Stibanose on leishmaniasis in hamsters.

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[2] Gellhorn, A., van Dyke, H. B., Pyles, W. J., and Tupikova, N. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 25.

Summary. A method for the routine evaluation of drugs for the therapy of visceral leishmaniasis in the hamster is described. No drug superior to Stibanose, Neostam or Neostibosan was discovered in tests of 27 other antimonials or of 158 additional metallic and non-metallic organic compounds.

15334

Effects of Inflammation (Turpentine Abscess) on Iron Absorption.

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These experiments with radioactive iron are concerned with iron absorption from the intestinal tract as influenced by infection. For many years pathologists have used the turpentine sterile abscess as a means of studying the response of experimental animals to a simple inflammatory reaction. Several dogs were accordingly studied in order to determine how such an inflammation might influence the uptake of labelled iron in single doses. It has been shown that the individual response to iron therapy is quite variable and necessitates control studies in the same subject.[1] The resulting data are summarized in Table I.

Experimental Data. Dog 40-149, an adult female mongrel fox terrier, had been maintained by periodic bleeding at an anemic level of blood hemoglobin for about 2½ years. The diet consisted of hospital table scraps. One ml of turpentine was injected in the axilla and the next day a test dose of 10 mg of iron labelled by radioactivity was given by mouth. Subsequent blood samples over a 2-week period showed an uptake of 4.1% of the dose. Control feedings of iron before and after the abscess period showed an average utilization of 29.5% of the dose. The methods used in determinations of labelled iron utilization have been previously described.[2,3]

Dog 41-164, an adult mongrel female terrier, had been kept anemic by repeated bleeding for a period of one year. The diet consisted of hospital table scraps. One ml of turpentine was injected subcutaneously in the left axilla and 2 days later a test dose of 38 mg of labelled iron as ferric ammonium citrate was given by gavage. Four % of the test dose was utilized in hemoglobin formation. A control feeding of the same amount of iron a month later showed an uptake of 17.6% of the test dose.

Dog 39-196, an adult female mongrel spaniel, had been maintained at a low level of hemoglobin by bleeding over a period of 2½ years, and splenectomized 14 months previous to this experiment. The diet was low in iron, and consisted mainly of white bread, salmon, and dried milk powder. One ml of turpentine was injected subcutaneously in the left axilla. Two days later a single dose of 38 mg of labelled iron was administered as ferric ammonium citrate by gavage. After 9 days 5.3% had been utilized for hemoglobin formation. In an attempted control experiment carried out a month later

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[1] Hahn, P. F., Jones, Edgar, Lowe, R. C., Meneely, G. R., and Peacock, Wendell, *Am. J. Physiol.*, 1945, **143**, 191.

[2] Hahn, P. F., Ross, J. F., Balfour, W. M., Bale, W. F., and Whipple, G. H., *J. Exp. Med.*, 1942, **75**, 221.

[3] Hahn, P. F., *Am. J. Physiol.*, 1944, **141**, 363.

TABLE I.
Iron Absorption by Dogs with Subcutaneous Turpentine Abscesses and During Control Periods.

Dog	Date 1943	Hct. %	Dose mg	Form	% absorption	Experiment type
40-149	6/17	13.4	9	$FeCl_2$	41.0	Control
,,	7/20	23.0	10	,,	4.1	Turpentine 1 day previous
,,	9/13	18.3	10	,,	18.1	Control
41-164	1/9	26.9	38	FAC	4.1	Turpentine 2 days previous
,,	2/8	28.6	34	,,	17.6	Control
37-196	1/9	22.4	38	,,	5.3	Turpentine 2 days previous
,,	2/8	23.4	34	,,	6.9	Control

(This animal had been splenectomized and was therefore liable to *Bartonella* infection. That this infection was present was later indicated by reaction to Mapharsen).

Balance sheet on 1/9/43 feeding of dog 37-196:

1/19 Hct. 35.2% estimated CV. = 265 Total circulating RaFe = 4720 c/m
2/3 ,, 28.7 ,, ,, ,, = 635

Radioactivity lost to circulation = 4085
Radioactivity found in 1900 ml blood removed in period 1/19 to 2/3 = 4045

This balance indicates that there was no additional absorbed and stored radio-iron to be used under the stimulation of blood loss.

6.9% of a similar test dose was utilized.

This dog subsequently showed a low rate of hemoglobin production on an adequate diet, a result consistent with an infection with *Bartonella canis,* a common finding on splenectomized dogs. This probability was further supported by a much more rapid rise in hemoglobin after administering 18 mg of Mapharsen by vein.

Balance sheet data in Table I show that on subsequent bleeding there was a quantitative accounting for the labelled iron in circulation, but no further mobilization of labelled iron from the tissues under the stress of the resulting anemia. This indicates that infection had prevented the absorption of iron.

Discussion. It seems apparent from these 3 experiments that inflammation accompanying the presence of turpentine in the subcutaneous tissues markedly reduces the ability of the dog to absorb iron from the gastrointestinal tract. These findings are of interest because of the frequent occurrence of anemias, often refractory to treatment, associated with infections and inflammatory disease. That the utilization of iron already in the body of dogs may be hindered by infection has also been reported by Robscheit-Robbins and Whipple,[4] and recently by Gibson and Finch (personal communication) in studies on human subjects using radioactive iron.

Summary. The radioactive isotope of iron shows that iron absorption from the gastrointestinal tract of the dog is impaired by the presence of a sterile abscess in subcutaneous tissue.

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[4] Robscheit-Robbins, F. S., and Whipple, G. H., *J. Exp. Med.,* 1936, **63**, 767.

15335

Electrocardiographic Changes in Hemorrhage and Ischemic Compression Shock.*

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(Introduced by Carl J. Wiggers.)
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Prolonged and severe posthemorrhagic hypotension, as in standardized procedures described in various communications from this laboratory,[1,2] produces an irreversible state. This is evidenced by the fact that reinfusion of all withdrawn blood only temporarily restores normal blood pressures; within intervals of ½ to 4 hours arterial pressures progressively decline and the animal dies. Evidence has been presented[3,4,5] that impairment of the myocardium is a factor in this unfavorable response to blood transfusion. If this is true, it is reasonable to expect that a methodical series of electrocardiograms taken during the course of such experiments might manifest some changes.

Ischemic compression shock as produced by Green *et al.*[6] likewise produces progressive circulatory failure which is benefited only temporarily by transfusion of blood or artificial blood substitutes. The changes in effective venous pressures and the reactions of the myocardium to equivalent venous pressures have not been reported.

Methods. Dogs, 10-17 kilos in weight, were anesthetized with morphine (ca 3 mg) and sodium barbital (ca 200 mg/kilo). Standardized hemorrhagic shock was produced in 9 dogs as in previously reported experiments. The animals were bled until mean arterial pressure fell to 50 mm and this pressure was approximately maintained for 90 minutes. At the end of this period pressure was reduced to and maintained at 30 mm for 45 minutes. All withdrawn heparinized blood was then reinfused and the onset of circulatory failure awaited.

Ischemic compression shock was produced in 9 dogs by the method described by Green *et al.*[6] Both hind legs were wrapped tightly from ankle to groin with stretched rubber tubing. This generally resulted in hypertension and dyspnea. After 6 hours the tube wrapping was removed. The arterial pressure fell rapidly to slightly subnormal levels and thereafter progressively declined to 50 or 40 mm Hg in 2 to 6 hours.

In both types of experiments complete series of electrocardiographic leads were taken at frequent intervals. Each series consisted of the 3 standard leads and 4 or 5 precordial leads using the central terminal of Wilson. In some experiments 3 "augmented leads" after the method of Goldberger were taken. Electrodes covered with a layer of cotton saturated with normal saline solutions were placed under the skin and left in place throughout the experiments. Mean blood pressure was recorded continuously from a femoral or carotid artery.

Results in Hemorrhagic Shock. With the exception of one dog, all showed normal electrocardiograms during the control period, but revealed more or less displacement of the S-T segment in several leads during the 50 and 30 mm periods. These electrocardio-

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* Supported by a grant from the Commonwealth Fund.

† Permanent address, University of Guayaquil, Ecuador.

[1] Wiggers, C. J., and Werle, J. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 604.

[2] Huizenga, K. A., Brofman, B. L., and Wiggers, Carl J., *J. Pharm. and Exp. Therap.*, 1943, **78**, 139.

[3] Wiggers, Carl J., and Werle, J. M., *Am. J. Physiol.*, 1942, **136**, 421.

[4] Wegria, Rene, Guevara Rojas, A., and Wiggers, Carl J., *Am. J. Physiol.*, 1943, **138**, 212.

[5] Wiggers, C. J., *Am. J. Physiol.*, 1945, **144**, 91.

[6] Green, H. D., Dworkin, R. M., Antos, R. J., and Bergeron, R., *Am. J. Physiol.*, 1944, **142**, 494.

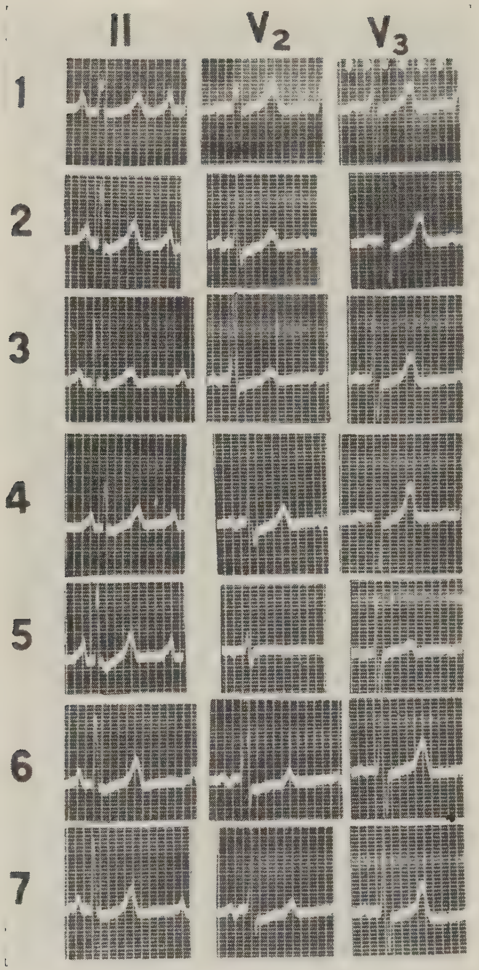

Fig. 1.

grams generally became normal again immediately after reinfusion, but again developed about the same degree of S-T displacement as arterial pressure began to fall. One dog showed no significant changes at any time. The S-T displacement usually varied from .055 to .25 mv. The greatest displacement usually occurred in leads V_2 and V_3, less often in leads II and III (standard), and rarely in lead I. In 5 of the 8 dogs, depression of the S-T segment was much greater during the period of initial hypotension than during circulatory failure following reinfusion and in 2 instances no deviation of the S-T segment occurred during post-infusion circulatory failure.

Sample records of leads II, V_2 and V_3 during the course of one experiment are reproduced in Fig. 1. It can be seen at a glance that the character of QRS changes during the course of the experiment. This is not remarkable or significant when we consider that the size and position of the dog's ventricles and their relation to the chest wall change as a result of withdrawal and reinfusion of blood and possible changes in the expiratory position of the thorax.[7] Whether this also enters into changes in the configuration of T waves in the upper 3 records must remain undecided at the present time. However, it is improbable that displacements of the S-T segment with peaking of the large T waves in records 4, 6 and 7 can be referred to factors other than functional changes in the myocardium. Curves 1 show normal controls; curves 2 and 3 were taken at the beginning and end of the 50 mm period of post-hemorrhagic hypotension. In both series the downward displacement of S-T is significant. Series IV at the end of the 30 mm period still reveals displacement and a peaked T wave, particularly in leads V_3 and V_4. Series V, taken within 15 minutes after reinfusion, shows no significant changes in QRS. The T waves are small and S-T depression is absent in the chest leads. Series VI was taken about ½ hour after reinfusion, while arterial pressure was still sustained. Marked depression of the S-T segment redeveloped particularly in leads V_2 and V_3, and T becomes larger and peaked in chest leads. Series VII was taken during the subsequent circulatory failure when arterial pressure had declined to about 60 mm Hg. Persistence of S-T depression and the large peaked T is obvious.

Conclusions. Constant displacement of the S-T segment in several leads (one exception) during the periods of severe hypotension indicates that functional myocardial changes occurred as a result of reduced coronary blood flow. The persistence of such changes after reinfusion, or their reappearance *before arterial pressure has declined* a great deal, strongly support the concept that functional

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[7] Harris, A. S., *Am. J. Physiol.*, 1945, **143**, 140.

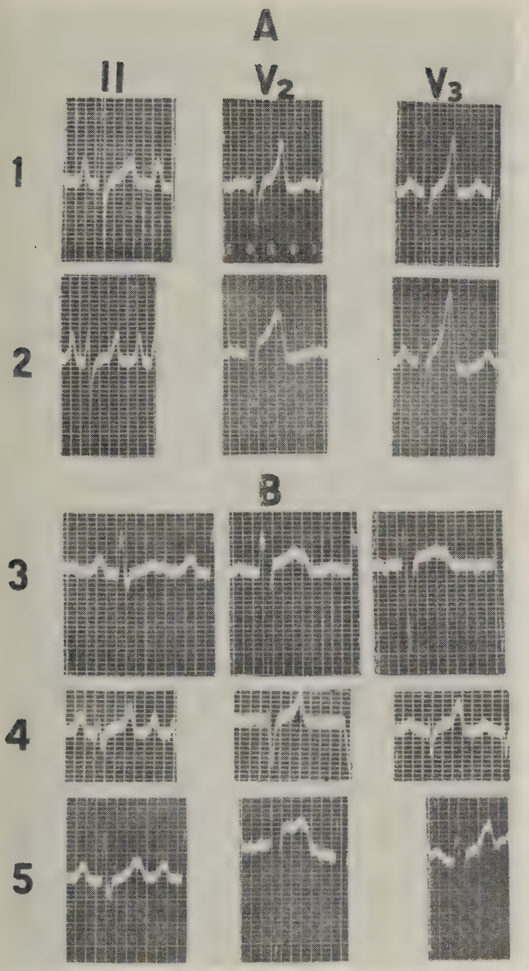

Fig. 2.

myocardial changes contribute to the subsequent circulatory failure.

Results in Ischemic Compression Shock. In 9 animals, to our surprise, significant S-T deviations generally appeared in several leads during the 6-hour period when compression tubes were kept on and while hypertension (140-210 mm Hg) existed. In 3 dogs the S-T segment was elevated and in 6, depressed (.05-.2 mv.) (In one dog the S-T segment reverted to normal and in another it increased progressively). Although dogs were fully anesthetized, painful stimulation was indicated by the augmented breathing and other reactions. It appears that such reflex stimulation in some way affects the myocardium, as indicated by S-T displacement. This is supported by the fact that upon removal of the tubing and release from painful stimulation, the S-T displacement was decreased or abrogated in 7 dogs. However, the displacement of S-T increased in 2 animals. As circulatory failure developed, the displacement increased in one dog only; in the others it remained nearly the same. If these electrographic signs are diagnostic of myocardial impairment it appears that the damage generally occurs during compression rather than after its release. As in hemorrhagic shock, the changes in T waves consisted chiefly of an increase in amplitude of T deflections and sharper peaks; inversion was never observed.

Typical changes in leads II_1, V_2, and V_3 from 2 animals during compression and decompression are reproduced in Fig. 2. In record 1, taken during compression, slight displacement of S-T is seen in leads II and V_2. Upon decompression, shown in record 2, the displacement becomes very marked in leads V_2 and V_3. Such reactions occurred in 2 dogs only. The typical effects in the other dogs are shown in curves 3 and 4. In curve 3, taken during compression, definite elevation of S-T is shown in leads V_2 and V_3. After decompression (curve 4) this displacement is abrogated.

In this connection the observations of Cicardo,[8] that the plasma potassium of femoral venous blood is definitely increased immediately after removal of a tourniquet (avg $11.2 < 15.2$ mg %), require correlation. The fact that characteristic displacements in S-T segments are not generally increased, but more often reduced after removal of the rubber tubes, suggests that the increased quantities of potassium which may enter the circulation after decompression are not always sufficient to be detectable by electrocardiographic leads.

Effects of Massage. Since the electrocardiographic changes during the course of circulatory failure did not alter significantly, the procedure was altered in the latter experiments by massaging the swollen limbs.

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[8] Cicardo, V. H., *Rev. d. l. Soc. Arg. de Biol.*, 1943, **19**, 511.

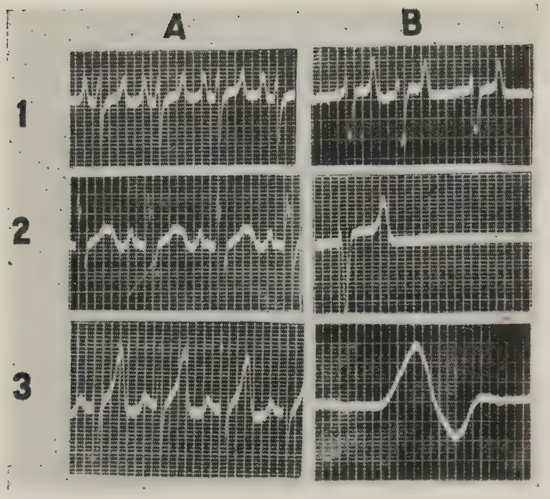

Fig. 3.

It was obvious from observations of arterial pressures that this hastened the downward course materially. As a rule, each period of massage decreased the heart rate materially. Thus, in several animals the following decelerations were observed: $204 > 160$, $136 > 96$, $220 > 180$, $160 > 60$ per minute. A drop of blood pressure attended each reduction in heart rate. Electrocardiograms showed a definite increase in S-T segment displacement as illustrated in curve 5, Fig. 2. One dog developed atrial fibrillation 32 minutes after massage, another showed a pronounced elevation of the S-T segment, and a third passed into ventricular fibrillation.

Fig. 3 illustrates changes in V_3 leads of 3 different animals. Record 1 shows atrial fibrillation with marked peaking of T waves; record 2 shows bradycardia of a dying heart, and record 3 broad deflection preceding ventricular fibrillation. Attention is called to the fact that ventricular fibrillation does not develop from a series of progressively accelerating beats, as after electrical stimulation or coronary occlusion, but is preceded by broad deflections similar to those reported as preceding fibrillation due to K or digitalis.[9,10]

We may therefore conclude on the basis of our experiments that while absorption of substances with a potassium-like action is not always detectable by electrographic technics when limbs are kept at rest, such substances are obviously present in tissues submitted to prolonged ischemic compression, and can be pressed into the circulation by massage (and possibly movements) in sufficient amounts to accelerate the production of circulatory failure. Electrographic studies strongly suggest that cardiac damage plays an important role, and indeed may be a cause of sudden death after massage. The importance of complete rest of damaged limbs is reemphasized.

Summary. Changes in configuration of records from chest leads paired with a central terminal and of the 3 standard electrocardiographic leads were studied during the course of standardized hemorrhagic shock and ischemic compression shock produced in dogs.

During post-hemorrhagic hypotension (50-30 mm Hg) displacement of the S-T segment in several leads occurred in 8 out of 9 animals. The greatest displacement usually occurred in leads V_2 and V_3; it occurred less often in leads II and III, and rarely in lead I. The electrocardiograms became normal again after replacement of withdrawn blood, but S-T displacement redeveloped and the T waves became large and sharp before arterial pressure had started to fall. The electrographic changes support the concept that myocardial changes develop in hemorrhagic shock. Similar changes in T wave and S-T segment in several leads—dominantly in V_2 and V_3—also occurred in ischemic compression shock. The alterations occur soon after limb compression by rubber tubes. A reflexogenic origin is postulated. In 7 out of 9 dogs displacement of the S-T segment decreased materially, or was completely abolished on removal of the compression tubes. The displacement increased in only one animal with development of circulatory failure. However, a prompt, marked increase in the S-T segment occurred when a bruised limb was massaged, and various serious cardiac disturbances (marked slowing, atrial and

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[9] Wiggers, Carl J., *Am. J. Physiol.*, 1930, **93**, 197.

[10] Wegria, R., Geyer, J. H., and Brown, B. S., *J. Pharm. and Exp. Therap.*, 1941, **71**, 336.

ventricular fibrillation) developed after vigorous massage. The results suggest that toxic substances with potassium-like actions are present in damaged tissues, that they do not enter the blood stream in sufficient amounts to be detectable when limbs are kept at rest, but that they may contribute materially to the circulatory failure and even lead to prompt cardiac death whenever limbs are massaged.

15336

Respiration of Heart Muscle Slices from Rats in the Terminal Stage of Hemorrhagic Shock.*

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Although venous return, cardiac output, and blood pressure are diminished in shock, it is usually assumed that the condition of the heart muscle plays no part in the development of severe states of shock. Reflex dilatation of the coronary vessels and the consequent re-routing of larger amounts of blood through the lungs tend to favor the oxygen supply of heart muscle. However, a period of relative anoxia of the cardiac muscle in the early stages of shock after hemorrhage may lead to an impaired ability of the muscle to consume oxygen. A fall in the rate of energy production of the heart muscle might, therefore, play a part in the reduction of cardiac output and of blood pressure seen in shock. The possibility that the rate of respiration of heart tissue, like that of liver tissue,[1] is depressed in hemorrhagic shock in the rat has, therefore, been examined in a short series of experiments.

Methods. Shock was produced in male rats of the Sprague-Dawley strain, weighing between 220 and 250 g, by slow hemorrhage from the tail vein and subsequent replacement of the blood volume according to the method of Sayers, Sayers, and Long.[2] In this procedure a prolonged state of severe shock is developed, and the animal dies, even though more than the original volume of blood lost by hemorrhage is replaced. When each animal was in the last stages of shock, several minutes before death was expected (as indicated by the gasping nature of the respiration), the heart was quickly excised, immersed in physiological salt solution until the blood had been pumped from the ventricles, and the great vessels were trimmed away. Thin slices of the heart muscle were prepared and collected in a moist chamber immersed in ice. Samples of the tissue weighing about 85 mg were distributed in Warburg vessels containing 1.5 ml of Krebs' physiological salt solution buffered with phosphate (0.017 M; pH 7.4). Parallel samples in duplicate were weighed into tared vessels and placed in the oven for drying to constant weight at 110°C. The percentage dry weight so obtained was used to calculate the dry weight of the tissue samples used for the measurement of respiration, and the rates of oxygen uptake were thus calculated in terms of the initial dry weight of the tissue. The oxygen uptake was measured in the Warburg apparatus, at 38°C, under an atmosphere of 100% oxygen, using 4 vessels, 2 without and 2 with added substrate. The substrates used were glucose (200 mg %) and sodium pyruvate (0.02 M). Addition of

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Yale University.

[1] Russell, J. A., Long, C. N. H., and Wilhelmi, A. E., *J. Exp. Med.*, 1944, **79**, 23.

[2] Sayers, G., Sayers, M. A., and Long, C. N. H., unpublished observations.

TABLE I.
Rates of Oxygen Uptake of Heart Muscle Slices from Normal Rats and from Rats in the Terminal Stages of Prolonged Severe Hemorrhagic Shock, in the Absence of Substrate and in the Presence of 0.02 M Sodium Pyruvate.

Substrate	Group	No. of animals	No. of determinations		Minutes 30	60	90
None	Control	9	18	Mean Q_{O_2}*	7.86±.34†	7.9±.3	7.14±.29
				P	.02	.05	.01
	Experimental	5	10	Mean Q_{O_2}	5.74±.34	6.22±.39	4.82±.38
Sodium Pyruvate (.02 M.)	Control	7	14	Mean Q_{O_2}	9.13±.35	11.3±.4	10.59±.24
				P	0.1	0.1	0.3
	Exp.	5	10	Mean Q_{O_2}	7.61±.36	9.7±.36	9.85±.35

* Cu mm of oxygen consumed per mg of initial dry weight per hour.
† Standard error of the mean.

0.5 ml of substrate solution or of physiological salt solution from the side bulbs of the vessels was made after an equilibration period of 10 minutes. Control observations were made on tissue slices prepared from the hearts of normal rats anesthetized with nembutal (4 mg per 100 g of body weight).

Results and Discussion. In 2 preliminary experiments it was found that added glucose depressed the oxygen consumption of normal cardiac muscle slices. There was a uniform elevation of the rate of oxygen uptake when sodium pyruvate was used as a substrate, and the remainder of the experiments were carried out in duplicate with this substrate. It was found that no additional information was obtained when the incubation was prolonged beyond 90 minutes, the decrement in rate of respiration being similar for cardiac muscle slices both with and without substrate. There was no significant difference in the initial dry weights of heart tissue samples from control and experimental animals. The percentage in the former was 22.1 ± 2.2 and in the latter it was 20.9 ± 2.9.

The results of a series of observations on control animals and on animals in the terminal stages of hemorrhagic shock are summarized in Table I. The rate of oxygen uptake, in the absence of substrate, of heart muscle slices from rats in shock is significantly less than the normal rate. This difference increases with increasing time of incubation, indicating that the respiration is also less well maintained than it is in heart muscle slices from normal rats. In the terminal stages of shock after hemorrhage, the rate of respiration of the heart muscle is, therefore, moderately depressed. If, however, this effect is to play a part in the development and maintenance of shock, it must occur at a much earlier time. Further experiments are necessary to determine how soon such an effect on cardiac respiration may develop.

The rate of oxygen uptake of heart muscle slices from rats in shock, in the presence of sodium pyruvate, is also less than the normal rate, but the observed differences in rate are not at any time significant. There is, therefore, no conclusive evidence of impaired ability of the heart muscle to utilize pyruvate or to maintain respiration at the higher rate attained after the addition of this substrate. It is possible, however, that a real difference in the rate of oxygen uptake of heart muscle from rats in shock and from normal rats might become apparent with a saturating concentration of pyruvate.

These preliminary observations on cardiac tissue metabolism in the terminal stages of hemorrhagic shock indicate that a further study of changes in cardiac metabolism during shock might be profitable. It is desirable to repeat these experiments on animals in earlier stages of shock and to supplement the studies on respiration with determination of rates of glycolysis and of the lactic acid and glycogen content of the heart muscle of animals in various stages of shock.

Summary. The rate of oxygen uptake of heart muscle slices from rats in the terminal stages of prolonged severe hemorrhagic shock is significantly less than normal in the absence of substrate. In the presence

of pyruvate (0.02 M) the rate of oxygen uptake is increased, but is still less than the normal rate. There is, therefore, no conclusive evidence of impaired ability of the heart muscle to oxidize pyruvate in these circumstances.

15337

Thiamin Deficiency and Susceptibility of Rats and Mice to Infection with *Salmonella typhi murium*.*

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It has been shown that rats and mice which are deficient in either riboflavin, vitamin A or biotin are markedly susceptible to infection with *Salmonella* (riboflavin: Kligler, Guggenheim and Buechler;[1] vitamin A: Kligler, Guggenheim and Henig;[2] biotin: Kligler, Guggenheim and Herrnheiser).[3] In vitamin A deficiency the resistance falls noticeably in late stages of the avitaminosis and is similar in extent to that of animals given adequate amounts of vitamin A but allowed only as much food as is ingested by animals on vitamin A-free diet. In riboflavin deficiency, on the other hand, a specific effect on the susceptibility of the animals to *Salmonella* is achieved. The susceptibility of riboflavin-deficient mice to *Salmonella* infection exceeds both that of paired isocaloric-fed mice on full vitamin diet and that of normal control mice. The effect of biotin deficiency is an even more specific one. In this case the increased susceptibility precedes the fall in the food intake and cannot, therefore, be due even partially to the latter.

Below we present experiments concerning the effect of thiamin deficiency on the susceptibility of rats and mice to *Salmonella* infection.

Methods. The rats and mice were taken from our breeding stock after weaning. The experimental diet consisted of 72 parts rice flour, 13 parts casein, 8 parts dried yeast and 4 parts salt mixture. After autoclaving for 6 hours at a pressure of 15 lb. 3 parts olive oil and 2 mg calcium pantothenate per 100 g food were added. Each rat was given 100 I.U. vitamin A and 4 I.U. vitamin D twice a week, the mice receiving half this amount. The control and paired-fed rats received in addition daily 20 γ thiamin, the control and paired-fed mice received 10 γ of thiamin daily.

The strain of *Salmonella typhi murium* employed for the production of the experimental infections was the same as that used in earlier experiments.

Results. I. Experimental infections of rats. Forty-eight animals were divided into 3 groups. Group A received food *ad libitum* without supplement of thiamin; group B was paired-fed with group A and received a supplement of thiamin; group C received food *ad libitum* and supplement of thiamin. The ingestion of food in group A was in the first 2 weeks of the experiment normal, but from the third week began to fall off gradually, until it rated in the fifth week of the experiment only one-half that of group C. The weights became constant at this stage. As rats of group A reached this stage of avitaminosis, all 3 groups were infected *per os* with 0.1 cc of a 24-hour broth culture of *Salmonella typhi murium* diluted 1:10 with milk. Three days later the animals were killed, and cultures were made from their

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* These studies were supported by a grant from the Palestine Endowment Fund, New York.

[1] Kligler, I. J., Guggenheim, K., and Buechler, E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 132.

[2] Kligler, I. J., Guggenheim, K., and Henig, E., *J. Hyg.*, 1945, **44**, 61.

[3] Kligler, I. J., Guggenheim, K., and Herrnheiser, H., *J. Inf. Dis.*, in press.

liver, spleen and mesenteric glands. The results are given in Table I. The thiamin-deficient and paired-fed rats proved markedly less resistant to *Salmonella* infection than the controls. Statistically this diminished resistance was found to be highly significant. The decrease of resistance was reflected both in the incidence and in the manner of distribution of the infection.

The similar response of the paired-fed and of the avitaminotic group shows the non-specific nature of the effect of thiamin deficiency. This is confirmed by the experiments in which rats in an early stage of avitaminosis (after 2 weeks of the deficiency diet) were infected, *i.e.*, before any decrease in food intake had occurred. As may be seen from Table I, hypovitaminotic rats are not more susceptible to infection than the controls.

II. Observations on experimental epidemics in mice. Four-week-old litter mates were taken directly from the mothers and divided into 3 groups, each group comprising 60 mice. Group A was kept on our thiamin-deficient diet, which was given *ad libitum;* group B was isocalorically paired fed to A with thiamin supplement; whereas group C received food *ad libitum* with thiamin supplement. Each group was divided into 4 cages, 15 mice per cage. At the start of the experiment one mouse in each cage was infected with 0.05 cc of a 24-hour broth culture of *Salmonella typhi murium.* Cultures were made from the liver, spleen and mesenteric glands of mice which died during the following fortnight. At the termination of this observation period the survivors were killed and examined similarly. The results are summarized in Table II. The table shows that the gain of weight of mice kept on a thiamin-deficient diet is significantly below that of the 2 other groups, although the food intake of the paired-fed group and of the control mice was practically the same. It is also evident that the thiamin-deficient mice showed a markedly higher rate of infection than the paired-fed and control mice. This enhanced susceptibility to infection on the part of the thiamin-deficient mice can only be attributed, therefore, to their thiamin

TABLE I.

Susceptibility of Rats in Various Stages of Thiamin Deficiency to *Salmonella typhi murium* Infection.

Nutritional condition	No. of rats	Weeks of experimental diet	Wt in g		No. infected	No. of rats with infection in			
			At start of exper.	At time of infection		Liver	Spleen	Liver and spleen	Mesenteric glands
Deficiency	16	4-5	27	44	15	13	11	11	14
Paired-fed	16	,,	27	61	15	13	13	13	13
Control	16	,,	27	90	9	3	4	2	7
Deficiency	30	2	27	38	3	1	2	0	2
Control	30	,,	27	53	3	1	1	1	2

deficiency.

A feeble effect of thiamin deficiency is already demonstrable, as the following experiment shows, on the eighth day of the observation period: 84 litter mates were divided into 2 groups. The animals were kept in cages which contained 21 mice each. One mouse in each cage was infected, as described above, and the entire mouse population was then treated in the manner of the former experiment, with the exception that the survivors were killed after 8 days. As may be seen from Table II, a difference in the incidence of infection in the thiamin-deficient group is already demonstrable at this early date. It is evident, however, that the mouse organism is still able at this stage to combat the invasion of bacteria from the mesenteric glands.

TABLE II.

Susceptibility of Mice in Various Stages of Thiamin Deficiency to *Salmonella typhi murium* Infection.

Nutritional condition	No. of mice*	Period of observation days	Wt in g		No. died†	No. alive and cultured	Total infected‡	No. of mice with infection in			
			Start of exper.	At time of culturing				Liver	Spleen	Liver and spleen	Mesenteric glands
Deficiency	56	15	11.7	12.5	36	20	20 (100)	16 (80)	16 (80)	15 (75)	20 (100)
Paired-fed	56	15	11.7	14.0	9	47	31 (66)	14 (30)	15 (32)	8 (17)	25 (53)
Control	56	15	11.7	14.1	8	48	37 (77)	14 (29)	17 (36)	10 (21)	32 (67)
Deficiency	80	8	11.2	11.6	1	79	59 (75)	6 (8)	8 (10)	6 (8)	56 (71)
Control	80	8	11.2	12.4	0	80	40 (50)	10 (12)	9 (11)	6 (8)	37 (46)

* The one initially infected mouse in each cage is not included in the number given.
† Out of a total of 54 mice which died the organs of 42 mice were cultured. In every case general infection with *Salmonella* was found.
‡ The numbers in parentheses show the percentage.

Discussion. The experiments and observations reported above provide an unequivocal answer to the question, whether thiamin deficiency affects resistance to *Salmonella* infection. When rats and mice are placed on a thiamin-deficient diet, they evince markedly increased susceptibility to enteric infection already within a few weeks.

There is a significant difference between the behaviour of the rat and of the mouse. Whereas in the case of the mouse the diminished resistance is exclusively attributable to the thiamin deficiency, the higher susceptibility of rats is due to the inanition which accompanies avitaminosis. The mice behave, in other words, as in the case of biotin deficiency,[3] whereas the rats behave as in the case of vitamin A deficiency.[2]

The effect of thiamin deficiency on resistance to infection has been studied by several authors. Badger, Masunaga and Wolf[4] observed a diminished resistance to rat leprosy in thiamin deficiency. A high susceptibility to experimental pneumococcal infection could be shown by Wooley and Sebrell[5] in mice and by Robinson and Siegel[6] in rats. The diminished resistance was found to be due to the avitaminosis *per se,* the concomitant starvation having no apparent influence. Thiamin-deficient rats show also a lowered resistance to infection by *Nippostrongylus muris.*[7] On the other hand, thiamin-deficient mice exhibit an increased resistance to infection with Theiler's virus and poliomyelitis.[8-11] Clark, Waisman, Lichstein and Jones[12] showed, however, in studies with *Macaca mulatta,* that this species, when deficient in thiamin, does not exhibit increased resistance to poliomyelitis virus. Further study of the effect of thiamin deficiency on the resistance of different species of animals to bacterial and viral infection seems, therefore, to be necessary.

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[4] Badger, L. F., Masunaga, E., and Wolf, D., *Pub. Health Rep.*, 1940, **55**, 1027.

[5] Wooley, J. G., and Sebrell, W. H., *Pub. Health Rep.*, 1942, **57**, 149.

[6] Robinson, H. J., and Siegel, H., *J. Inf. Dis.*, 1944, **75**, 127.

Summary. (1) Rats and mice kept for a few weeks on a thiamin-deficient diet show diminished resistance to oral *Salmonella typhi murium* infection.

(2) In rats the lowered susceptibility is secondary to inanition. On the other hand, in mice the diminished resistance to *Salmonella* infection is a primary result of the thiamin deficiency.

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[7] Watt, J. Y. C., *Am. J. Hyg.*, 1944, **39**, 145.

[8] Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *Am. J. Med. Sc.*, 1943, **205**, 465.

[9] Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *Science*, 1943, **97**, 207.

[10] Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **80**, 257.

[11] Rasmussen, A. F., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Inf. Dis.*, 1944, **74**, 41.

[12] Clark, P. F., Waisman, H. A., Lichstein, H. C., and Jones, E. S., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 42.

15338

Rh Antigen and Hapten. Nature of Antigen and Its Isolation from Erythrocyte Stroma.

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(Introduced by A. L. Bloomfield.)

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Introduction. The demonstration of the role played by the Rh factor of human erythrocytes in isoimmunization[1,2] leads to speculation concerning the usefulness of the isolated antigen or hapten in modification of the sensitized state. This would be particularly important in hemolytic disease of the newborn, in which maternal anti-Rh antibodies enter the foetal circulation and produce accelerated hemolysis of the infant's erythrocytes. It seems possible that a preparation of Rh antigen could be used to neutralize the anti-Rh antibodies in the maternal circulation and tissue. If sufficient antigen could be injected at regular intervals antibody formation, which apparently has some natural limitation, could be overwhelmed and the subject kept in a state of desensitization until pregnancy is terminated. If the isolation and purification of the Rh antigen can be carried to the point of separation of the Rh hapten from the associated protein it would presumably no longer possess antigenic qualities but would still be capable of neutralizing the antibodies.

Belkin and Wiener[3] have shown by serum inhibition experiments that the Rh factor as well as the A and B substances can be demonstrated in roughly quantitative amounts in the stroma of human erythrocytes. We

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[1] Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

[2] Levine, P., Katzin, E. M., and Burnham, L., *J. A. M. A.*, 1941, **116**, 825.

[3] Belkin, R., and Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 214.

TABLE I.
Selective Inhibition of Anti-Rh_0 Serum by Stroma from Rh-Positive Cells.

Dilutions of 10% stroma suspension	1-1	1-2	1-4	1-8	1-16	1-32
Stroma from Rh+ cells	0	0	0	0	+	+
” ” Rh— ”	+	+	+	+	+	+

have undertaken to find means of separating the Rh antigen from other components of erythrocyte stroma by chemical and physical methods and to obtain information as to the nature and properties of the antigen.

Material and Methods. 1. General methods for production of post hemolytic residues or stroma. The erythrocytes used were obtained from citrated blood after centrifugation and removal of the plasma.* Usually only group O cells were used, but on some occasions cells from 50 to 100 untyped bleedings were pooled without regard to blood group. On some occasions stroma was prepared from Rh positive and from Rh negative cells, but in general large lots of cells were used without regard to Rh typing. Most of the leucocyte and platelet layer was removed from the centrifuged blood before the cells were hemolyzed.

Hemolysis was accomplished by adding 8 to 10 volumes of distilled water which had been chilled to a temperature of 5°C. In the initial preparations the solution was then fed through a Sharples supercentrifuge. The rate of flow depended on the model and speed of the centrifuge employed. The red jell-like material collected by centrifugation was sometimes resuspended and sedimented again in order to remove more of the remaining hemoglobin.

The material was then kept in solution at a pH between 7 and 8 or dried by lyophylization. In either state it was found to retain most of its potency for weeks, when stored at 2°C.

Suspensions of the dried material, or fractions thereof, were made in 10% concentration in isotonic saline, or in phosphate buffer pH 7.7 ionic strength .2. An even suspension of fine particles was obtained by grinding the suspension in a mortar. In later purified preparations the material dispersed quite readily upon mixing and standing a few hours.

2. Methods used in detecting the presence and concentration of the Rh antigen in erythrocyte stroma and fractions thereof. The anti-Rh serum used in the inhibition experiments agglutinates approximately 85% of human Rh-positive bloods and has a titer of 400. It was used in dilution of 1-20 with normal saline.

Serial dilutions were made of the stroma suspensions, which were then mixed with equal volumes of the constant dilution of the anti-Rh serum. The stroma serum mixtures were incubated for one hour at 37°C. Following incubation the tubes were centrifuged to obtain a relatively clear supernatant fluid. One drop from each tube was incubated for ½ hour with one drop of a 2% suspension of Rh positive cells which had been shown to give a strong reaction with the anti-serum. Final observation for the presence or absence of agglutination was made after centrifugation and inspection of the erythrocytes under low power magnification. The inhibition titer of the stroma preparation was read as the highest dilution of a 10% suspension which inhibited agglutination by the constant strength of the anti-Rh serum.

The same technic was used in quantitating the A and B substances in stroma preparations made from pooled untyped cells, except that a constant strength of groups B and A sera was used. The sera had titers of 3000 and were used in dilutions of 1-20.

Inhibition of anti-Rh serum with stroma from Rh-positive and Rh-negative erythrocytes. Our first experiments were designed to demonstrate that the inhibition of anti-Rh serum by stroma from Rh-positive cells was not due to a nonspecific effect. Typical results of such observations are recorded in Table I.

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* We are grateful to the Cutter Laboratories of Berkeley, California, and especially to Mr. Virgil Herring, for the red cells and for the first stages in their processing.

TABLE II.
Sensitivity to Destruction of Rh Factor in Stroma by Heat.

Dilutions of 10% stroma	1-1	1-2	1-4	1-8	1-16	1-32	1-64
Control (unheated)	0	0	0	0	0	+	+
56°C 15 min.	+	+	+	+	+	+	+
100 15 ”	+	+	+	+	+	+	+

TABLE III.
Heat Destruction of Rh Factor with Moisture in Absence of Oxygen.

Dilutions of stroma (10%)		1-1	1-2	1-4	1-8	1-16
Control	no heat	0	0	0	+	+
Tube No. 1, vacuum	56°C 5 min.	0	0	0	+	+
” No. 2, air	” 5 ”	0	0	0	+	+
” No. 3, moisture	” 5 ”	+	+	+	+	+

Observations on the stability of the Rh factor in Erythrocyte Stroma. 1. Heat stability of the Rh factor in stroma. One of the characteristics of an antigen is its thermal stability (or instability). The results of such tests on the Rh antigen are given in Table II.

Further observations have indicated that the antigen is completely inactivated according to our methods of testing by exposure to 56°C for 5 minutes.

Stroma preparations containing in addition to Rh also the factors inhibiting group A and B sera showed no loss of these properties upon heating when the Rh antigen had been completely inactivated.

2. Effect of the presence of air and moisture on the heat stability of the Rh antigen. Aliquot portions of active stroma were lyophylized and sealed in 3 small evacuated tubes. The first tube was kept evacuated, while the contents of the second were exposed to air. To the third tube, the requisite amount of saline was added without breaking the vacuum. The saline had been previously boiled to remove dissolved oxygen, and then cooled.

All 3 tubes were then exposed to 56°C for 5 minutes. The results of serum inhibition experiments with samples from these tubes are shown in Table III.

The Fractionation of the Stroma. Red cell stroma suspensions have been prepared in a variety of ways with a corresponding variety of results.[4,5,6] It was soon apparent to us that because of the extreme sensitivity of the Rh antigen, chemical methods of fractionation would not be as satisfactory as physical methods, at least in the early stages of the separation.

Following the method of Jorpes,[6] we prepared the stroma suspensions by adjusting the pH of the original solution of hemolyzed red cells to 5.5-6.0, at which a heavy, easily separated precipitate was formed. This was washed several times at pH 5.5-6.0 to remove most of the adsorbed hemoglobin. When it was redissolved at pH 7-8 we found that a cloudy solution was produced from which no sediment would settle upon standing. However, when this solution was centrifuged at 50,000 G a material (elinin) was sedimented, leaving a clear supernatant from which a heavy precipitate could again be thrown out by readjustment of the pH to 5.5. It corresponds to the protein which Jorpes has called stromatin, which name we retain. Stromatin so prepared exhibits no inhibition of the anti-Rh serum.

The material (elinin) removed from the above mentioned cloudy solution by centrifugation at 50,000 G dissolves easily at pH 7.5-8.0, producing a milky solution which shows very marked streaming double refraction. Since this material is very different from stromatin and can be characterized by its physical, chemical and biological properties we propose to call it elinin.

The approximate yield of stroma was about 1-2 g per 250 cc of red cells, of which 35 to

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[4] Ponder, E., The Mammalian Red Cell and the Properties of Haemolytic Systems. Berlin: Borntraiger, 1934.

[5] Segurdsson, B., *J. Exp. Med.*, 1943, **77**, 315.

[6] Jorpes, E., *Biochem. J.*, 1932, **26**, 1488.

40% is elinin and the rest stromatin.

The ability to inhibit the anti-Rh serum was found only in the elinin fraction which also possesses a concentration of the A and B substance 4 to 5 times greater than does stromatin.

The crude stroma suspension contains about 20% lipids extractable by 3:1 alcohol:ether. The elinin contains 40-50% lipids extractable by the same solvent. The nitrogen and phosphorous analyses are as follows:

	Prep. I		Prep. II	
	%	%	%	%
Stromatin	N 12.3	P 0.42	N 12.1	P 0.25
Elinin	N 9.1	P 1.0	N 8.1	P 1.0

Neither of these preparations can yet be said to be pure.

Electrophoresis† of a .1% solution of elinin at 2°C in phosphate buffer pH 7.8, ionic strength .2, showed a main component (about 80% of the total) having an average mobility of 6×10^{-5} cm^{-2} $volt^{-1}$ sec^{-1} (negatively charged), and a faster component (about 15% of the total) having a mobility of about 8×10^{-5}. The partial specific values of elinin at 20° is about 0.83.

The samples of elinin so far prepared have exhibited inhibition titers for the anti-Rh serum as high as 1:32. On a number of occasions we have succeeded in separating from the elinin an ether-soluble material which exhibited inhibition titers for the anti-Rh serum as high as 1:128. But heretofore we have not been able to reproduce this extraction consistently. The ether-soluble factor appears to be relatively thermostable, *i.e.*, it is not inactivated by 60 minutes heating at 56°C. Part of the original thermolability of elinin is returned upon recombining the protein from which the ether-soluble material had been removed, with the ether-soluble fraction.

When elinin is prepared from pooled untyped blood it exhibits relatively high inhibition titers (1:512) for the standard anti-A sera and a correspondingly lower inhibition titer (1:32) for the standard anti-B sera. In the ether-soluble fraction from elinin the inhibition of anti-A serum was reduced (1-16) while the inhibition of anti-Rh serum was increased.

Summary. 1. It has been demonstrated that the Rh antigen is quite different in character from the A and B substances as found in stroma from human erythrocytes. The Rh factor is particularly sensitive to thermal inactivation and is destroyed by a few minutes exposure to 56°C while the A and B substances remain intact. The inactivation by heat in the presence of moisture and the absence of oxygen suggests protein denaturation as the basis for its destruction.

2. Erythrocyte stroma has been fractionated into 2 parts; (1) a protein previously described and called stromatin, and (2) a new lipoprotein which we call elinin. The Rh specificity is associated with the elinin.

3. An ether-soluble fraction has been separated from elinin which is characterized by a higher content of the Rh factor and a lower content of A and B substances. The Rh substance present in the ether solution which inhibits the anti-Rh serum showed an increased thermostability.

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† The electrophoresis was done by Dr. C. H. Li, of the Institute for Experimental Biology of the University of California, Berkeley, California, to whom we express our thanks.

15339

Portal Vein Ligation and the Eck Fistula in the Rat.

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Limited though it is, the Eck fistula has been of real value in the study of liver function since its development by N. V. Eck.[1] Many problems of nutrition, detoxication and hepatic inactivation may well be studied by the judicious use of animals in which the blood from the gastro-intestinal tract is diverted from its normal pathway through the liver. Unfortunately the Eck method of producing a porto-caval anastomosis has so far been applicable to only the larger laboratory animals in which even then the surgery has not been easy, and mortality is often high. Furthermore, the inherently proper animal for many problems could not be prepared.

In the search for a method for studying the hepatic inactivation of steroid hormones 2 procedures have been adapted or devised by either of which the widely used laboratory rat may be prepared to function in a manner similar to the Eck fistula dog. These operations require few special instruments and may be done with speed and moderate mortality. The first is a 2-stage ligation of the portal vein. The second is a porto-caval anastomosis comparable to that done on the dog. The 2 methods as well as the "reverse" Eck fistula are described separately.

Portal Vein Ligation. If the portal vein is completely ligated at one operation the rat dies of circulatory failure within one or 2 hours, apparently due to the reduced effective blood volume incident to the accumulation of blood in the abdominal viscera. If the vein is only partially ligated, however, at a first operation the increased pressure in the portal system stimulates the development of collateral pathways so that a subsequent complete ligation of the vessel is tolerated. The second similar operation is hazardous because of the severe hemorrhage resulting from the difficult process of dissecting away adhesions involving intestines and liver. Dragstedt[2] solved this problem by placing a second ligature at the time of the first operation and securing the ends of the thread on the inner surface of the body wall. His second operation was done easily by opening the abdomen, retrieving the anchored ends of the ligature and pulling the knot down on the vein.

The method here described may be best understood by reference to Fig. 1. An extensive mid-line incision of the abdominal wall is carried to or above the tip of the xiphoid process. With the viscera retracted to the left, the vessels involved are made visible, as illustrated. A thread is passed by curved forceps or the blunt end of a curved needle around the portal vein, at *a* in the figure. Only moderate care is needed to exclude the hepatic artery and common bile duct. A wire about 1 mm in diameter, gauge 16 or 18, is laid along the vein and the ligature formed by the thread which encloses both the vein and the wire is closed firmly. The wire is then withdrawn. The size of the effective lumen of the vein is thus approximately that of the wire employed. This partial ligation is apparently sufficient to stimulate the development of collateral circulation. A second ligature is placed around the vein at the same level and at the same operation. The knot used is the "surgeon's" or friction knot. It is left so loose that it offers no restriction to blood flow. The ends of the thread are carried through the lateral body walls in approximately the same transverse plane as the portal vein. The abdominal incision is closed by a single series of interrupted sutures. The extruding ends of the ligature are secured to the skin surface by a liberal application of an acetone

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[1] Eck, Nikolai V., *Voenno-meditsinskii Zhurnal,* 1877, **130**.

[2] Dragstedt, Lester R., *Science,* 1931, **73**, 315.

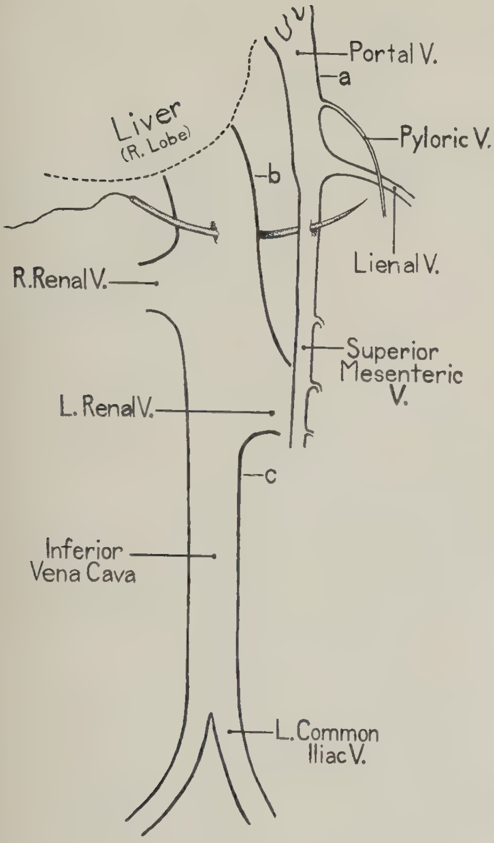

Fig. 1.
Portal vein ligation and Eck fistula in the rat.

solution of cellulose acetate. The thread and hair are well matted together. Heavy adhesive tape is passed around the body covering these areas to prevent the rat from pulling the threads prematurely. A strip of screen wire between layers of tape prevents the removal of the bandage by the animal. Four or 5 days later the tape is removed, the ligature ends exposed and pulled in such a manner that the "surgeon's" knot is tightly closed around the portal vein. This maneuver constitutes the second stage of portal ligation. Should the animal die within a few hours, it is highly probable that the first ligature had been too loose to stimulate development of collateral circulation. The ligature ends may be cut off, or by protecting them for 4 more days, the thread may be pulled completely from the body after some gentle manipulation. This last procedure was suggested by the work of Brunschwig.[3] This entire operation is practically bloodless. The rat is subjected to little more trauma than that of a laparotomy.

Porto-Caval Anastomosis (Eck fistula). Well deserved credit is given Nikolai V. Eck for developing the first surgical anastomosis between the portal venous system and the inferior vena cava. He, impelled by a desire to study liver function, developed a technic which he applied to 8 dogs. Of the 8, one did not survive 24 hours, 6 lived 2 to 7 days, and one recovered completely and lived in the laboratory for 2½ months when, by some negligence, it was allowed to escape. Dr. Eck's published report gives no indication that he used his dogs in any experimental study, nor that he proved the efficacy of the operation, but he implies that autopsies of those that died showed patent fistulae in most cases. Apparently military service interrupted his work at that point. At any rate, he believed that he had demonstrated that the operation was feasible, and that life is possible in an animal in which the liver is not the first recipient of the blood from the gastro-intestinal tract. The method was essentially that of securing the 2 veins to each other with 2 longitudinal rows of sutures and then making an incision between them.

Fishback[4] reviews the literature pertaining to the methods and modifications of the Eck fistula in his report describing the use of a cutting suture in making the anastomosis. Angle[5] described a special instrument suitable for the dog which clamped the 2 veins without occluding either in such a manner that the anastomosis could be made with a minimum loss of blood. Klages[6] made the Eck fistula applicable to the rabbit, by causing adhesions to develop between the 2 veins by a first operation, and later, by forming a window between them through the adherent area. Blakemore and Lord[7] have recently reported the highly successful application of the vitallium tube non-suture technic to the production of a porto-caval anastomosis in a number of patients with severe ascites due to portal bed block.

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[3] Brunschwig, Alexander, *Surgery*, 1945, **17**, 781.

[4] Fishback, F. C., *Ann. Surg.*, 1927, **86**, 436.

[5] Angle, Lewis W., *Am. J. Surg. n. s.*, 1933, **19**, 347.

[6] Klages, F., *Arch. f. Exp. Path. u. Pharm.*, 1938, **188**, 489.

The anastomosis between the portal venous system and the inferior vena cava here described is done in 2 stages. For the first operation, an extensive mid-abdominal incision is made and the viscera are retracted to the left, exposing the venous systems concerned, as shown in Fig. 1. A ligature is placed about the portal vein, at *a* in the illustration, formed with a "surgeon's" knot, but not closed. The ends of the thread are carried through the lateral body walls in preparation for tightening a few days later. In preparation for joining the veins the peritoneum is stripped from the vena cava in the neighborhood of the right renal vein where the vena cava and the superior mesenteric veins may be easily approximated. One of the smallest curved needles available is used to make the anastomosis. An artery suture with attached taper point needle, size 6-0 has been satisfactory, though the small cutting needle commonly found in eye sutures of comparable size offers one advantage. It makes larger punctures in the veins and thus offers more assurance of a successful anastomosis. The increased hemorrhage can be controlled. The needle is passed through the inferior vena cava, as shown in the illustration, and then through the adjacent superior mesenteric vein, piercing the lumina of both, and the suture is tied firmly. The suture includes a substantial part of the walls of the 2 veins. The fistula between the 2 veins should develop here to a functional size within a few days. There is very slight loss of blood from the punctured vena cava but precautions may well be taken to control loss from the mesenteric vein. The abdominal incision is closed and the ends of the delayed portal ligature fixed to the skin and protected as described for the portal ligation operation. Four or 5 days later this previously prepared ligature around the portal is tightened. If an anastomosis was not produced the animal will die within a few hours.

The "Reverse" Eck Fistula. Only a few modifications of the above procedures are necessary to produce the so-called "reverse" Eck fistula by which blood from the inferior vena cava is carried through the liver by way of the portal system. The anastomosis is made in the same manner as above. The delayed ligature is, however, placed on the vena cava above the renal veins (*b* in Fig. 1). If it is desired to study the effect of the liver on blood-carried products of the kidneys, adrenals, or gonads more specifically a ligature may be placed, at the time of the first operation, on the inferior vena cava below the renal veins (*c* in Fig. 1). This ligature is well tolerated and minimizes the possibility of the return of blood from the above mentioned organs to the heart by general collateral circulation.

The possibility exists that the effectiveness of any of the 3 operations described may be reduced by the post-operative development of venous channels around the ligatures. For example, an adhesion between the intestine and the liver may act as a bridge to carry venous blood directly to the liver. That this is not likely to be a serious hazard is indicated by the striking and generally consistent results already attained in studies of 2 different liver functions by the method. In the "reverse" Eck fistula enlarged collateral pathways around the ligature of the vena cava have been found. How serious this factor may be is not yet apparent. Obviously, a complete evaluation of the procedures described here must await their extensive use in experimental studies.

This single suture method of making a venous anastomosis was first suggested by Dr. John Lawther. In fact, he made the first attempt to carry it out.

All manipulations of the animal involved in the above operative procedures have been carried out under ether anesthesia. Nonsterile operative technic is apparently satisfactory for the healthy rat. The time required to do these operations is not excessive, for one operator working alone can perform

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[7] Blakemore, A. H., and Lord, J. W., *Ann. Surg.*, 1945, **122**, 476.

3 or 4 per hour. Efficient assistance can reduce this time considerably. Although surgery is easier on large adult rats, the Eck fistula has been produced on some rats weighing 80 g.

Summary. Methods have been described for producing in the rat, (a) a simple ligation of the portal vein, and (b) an anastomosis between the inferior vena cava and the portal venous system. By the use of the latter procedure, either a "direct" or an "indirect" Eck fistula may be produced. These technics should enhance the value of the laboratory rat in liver function studies.

15340 P

Number of Peyer's Patches in Mice Belonging to High and Low Mammary Tumor Strains.

Margaret A. Kelsall.* (Introduced by C. C. Little.)

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Peyer's patches are aggregates of lymphoid follicles in the mucosa of the small intestine. Their distribution and structure in the mouse are essentially similar to descriptions given for man, with the exception that 1 to 3 patches are usually present in the duodenum of the mouse whereas in man they are found only in the lower part of the small intestine.

A marked difference in the number of Peyer's patches in 2 strains of mice suggested a study to determine whether there was any significant difference in the amount of lymphoid tissue in the small intestine of mice belonging to high and low mammary tumor strains. One of the many functions assigned to the lymphocyte is that it has a part in tumor growth, the explanation of this function ranging from the conclusion that lymphoid tissue is a factor in immunity[1] to the contradiction that it has no part in tumor growth.[2]

Mice used were of both sexes and varied in age from 4 weeks to 1.5 years. The animals were not fed for 2 days prior to killing in order that the small intestine might be contracted, thereby making the Peyer's patches more conspicuous. The mice were killed by etherization; the small intestine and cecum were removed and fixed in a 10% formol solution, for 2 days, after which the intestines were washed thoroughly. The lymphoid nodules comprising the patches make the wall of the intestine considerably thicker, and as a result these areas protrude and are easily visible macroscopically.

The 5 strains may be divided into a low group, having an average of approximately 6.3 Peyer's patches, and including only strain C57 black; an intermediate group, having 8 to 9 patches and including strains C, Swiss, and dba; and a high group including C3H mice, which have a mean of 10.7 Peyer's patches (Table I). The differences between strain C57 black and the other strains were significant according to the X^2 test for goodness of fit. When data from C57 black mice were used for the theoretical distribution, strains C3H, dba, and Swiss had an X^2 above 70; the X^2 for C57 black and C was above 38, which gives a probability of approximately one in a million and indicates that this difference is also significant.

The difference in frequency distribution between strain C3H and the intermediate group is also significant, the X^2 for differences between C3H and dba being over 70. Additional data are necessary to establish significant differences between dba, Swiss, and C strains. The probability of a difference between dba and Swiss is only one in

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* The author is a recipient of a Finney-Howell Research Fellowship.

[1] Murphy, J. B., and Morton, J. J., *J. Exp. Med.*, 1915, **22**, 204.

[2] Sittenfield, M. J., *J. Cancer Res.*, 1920, **105**, 117.

TABLE I.
Range and Frequency Distribution in the Number of Peyer's Patches in the Small Intestine of 5 Inbred Strains of Mice.

Strain	No. of Peyer's patches											Total	Mean	St. dev.	St. error
	4	5	6	7	8	9	10	11	12	13	14				
C57Blk.	28	52	68	54	25	18	3	0	1	0	0	249	6.3	1.5	.09
C	1	2	15	24	24	21	14	8	2	1	0	112	8.2	1.7	.16
Swiss	2	6	8	34	36	20	21	11	3	0	0	138	8.3	1.7	.15
dba	0	2	9	22	55	28	20	13	4	3	1	157	8.6	1.6	.13
C3H	0	0	1	1	6	20	25	26	18	13	4	124	10.7	1.5	.14

a 100. The means for strains Swiss and C were 8.3 and 8.2 respectively, a difference that is insignificant, with a probability of only one in 10.

No differences were found between sexes within a strain, and the only difference in size observed was that most patches in the C3H mice were larger.

A direct correlation exists between the amount of intestinal lymphoid tissue, as measured by the number of Peyer's patches, and the incidence of spontaneous mammary tumors. Strain C3H has an incidence of mammary tumors in approximately 95% of the breeding females. Strain dba, which follows C3H in the number of Peyer's patches with a mean of 8.0, has a tumor incidence of approximately 51% in virgin females and 85% in breeding females.[3] Strains C and Swiss have fewer Peyer's patches and a lower incidence of mammary tumors. In strain C57 black, mammary tumors are extremely rare.[4]

There may be no functional relationship between the Peyer's patches and the mammary gland; however, several facts have been established that prove there is a functional relationship between the lymphoid tissue of the body as a whole and lactation. Emmel, Weatherford, and Streicher[5] have shown that lymphocytes pass in great numbers from the blood into the ducts of the mammary gland during lactation in the rat. In addition, the reticulo-endothelium of the lymphoid organs, including that in the Peyer's patches, is a source of monocytes, cells considered to be the colostrum corpuscles when occurring in the milk and containing fat globules. Quantitative differences in lymphocytes and monocytes may contribute to tumor growth, but functional differences in the lymphoid systems as a whole need to be established before any conclusions are warranted.

Summary. Line C57 black mice has a mean of 6.3 Peyer's patches; strains C, Swiss, and dba, means from 8 to 9; and strain C3H, a mean of 10.7. Differences between these groups are significant, according to the X^2 test. There is a direct correlation between the number of Peyer's patches and the incidence of spontaneous mammary tumors in these 5 pure lines of mice.

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[3] Little, C. C., *Biol. Lab. Mouse,* 1941, 248, Blakiston, Philadelphia.

[4] Cloudman, A. M., *Biol. Lab. Mouse,* 1941, 168, Blakiston, Philadelphia.

[5] Emmel, V. E., Weatherford, H. L., and Streicher, M. H., *Am. J. Anat.,* 1926, **38,** 1.

15341

A Quantitative Method for the Study of Chromatolysis.*

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The chromatolytic changes which follow section of the axon and certain other procedures consist in a continuous spectrum of cell alteration from the normal cell to the heavily chromatolyzed and even the ghost cell. To compare the degree of chromatolysis in 2 nuclei or to relate the chromatolytic changes with time, it is imperative that the spectrum of change be broken up into arbitrary and well-defined stages of a convenient number in order that standard quantitative procedures may be applied. Cognizance of this need led Dolley[1] to erect a series of 13 categories into which cells affected by shock and by exercise may be grouped. His method is not suitable for our purpose, however, for it is concerned with nuclear changes as much as with the changes of the Nissl bodies and was designed for rather a different purpose. The following method has been used in this laboratory with success and is recommended as being both simple and adequate.

The sequence of cytoplasmic changes which constitute the axon reaction is as follows: The Nissl bodies, normally abundant and square-cornered, become rounded, more lightly staining and begin to break down to a fine powder in the central areas of the cell. This fractionation then extends out to the periphery leaving the cytoplasm devoid of any blue-staining substance other than the finely-powdered material. Concomitant with the later stages is a shifting of the nucleus from its normal central position to the periphery of the cell. In addition to these changes, there are frequently present in cell groups in which chromatolysis has been induced, cells which have acquired more heavily-staining properties than the normal cell. These hyperchromic cells which have been noticed by many authors, range from a stage which shows the normal cell structure with the Nissl bodies more heavily stained to pyknotic heavily stained cells in which the cell contents are indistinguishable because of the depth of the stain. The physiological significance of this change is still a matter of conjecture. From our experience we are led to view such hyperchromic cells as bearing an opposite relation to the normal cell as does the chromatolyzed, and have so dealt with it in the categorization to be described below. It should be noted that Dolley (loc. cit.) follows the view that the hyperchromic cell is undergoing changes preliminary to chromatolysis. For this possibility we can find no evidence in our ob-

TABLE I.
Criteria Defining Cell Types.

B Pyknosis	A Hyperchromatism	N Normal	1 Mild chromatolysis	2 Medium chromatolysis	3 Severe chromatolysis
Shrunken, intense staining. Nucleus and cytoplasm obscured. Fig. 1,a.	Nissl particles larger, more heavily staining than normal. Fig. 1,b.	Nissl particles discrete, square-cornered. Moderately intense stain. Fig. 1,c.	Nissl particles breaking up in center of cell. Normal Nissl pattern in periphery. Fig. 1,d.	Nissl particles uniformly powdery throughout cell. Nucleus central. Fig. 1,e.	Nissl particles uniformly powdery. Nucleus peripheral. Fig. 1,f.

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Dolley, D. D., *J. Med. Res.*, 1911, **20**, 285.

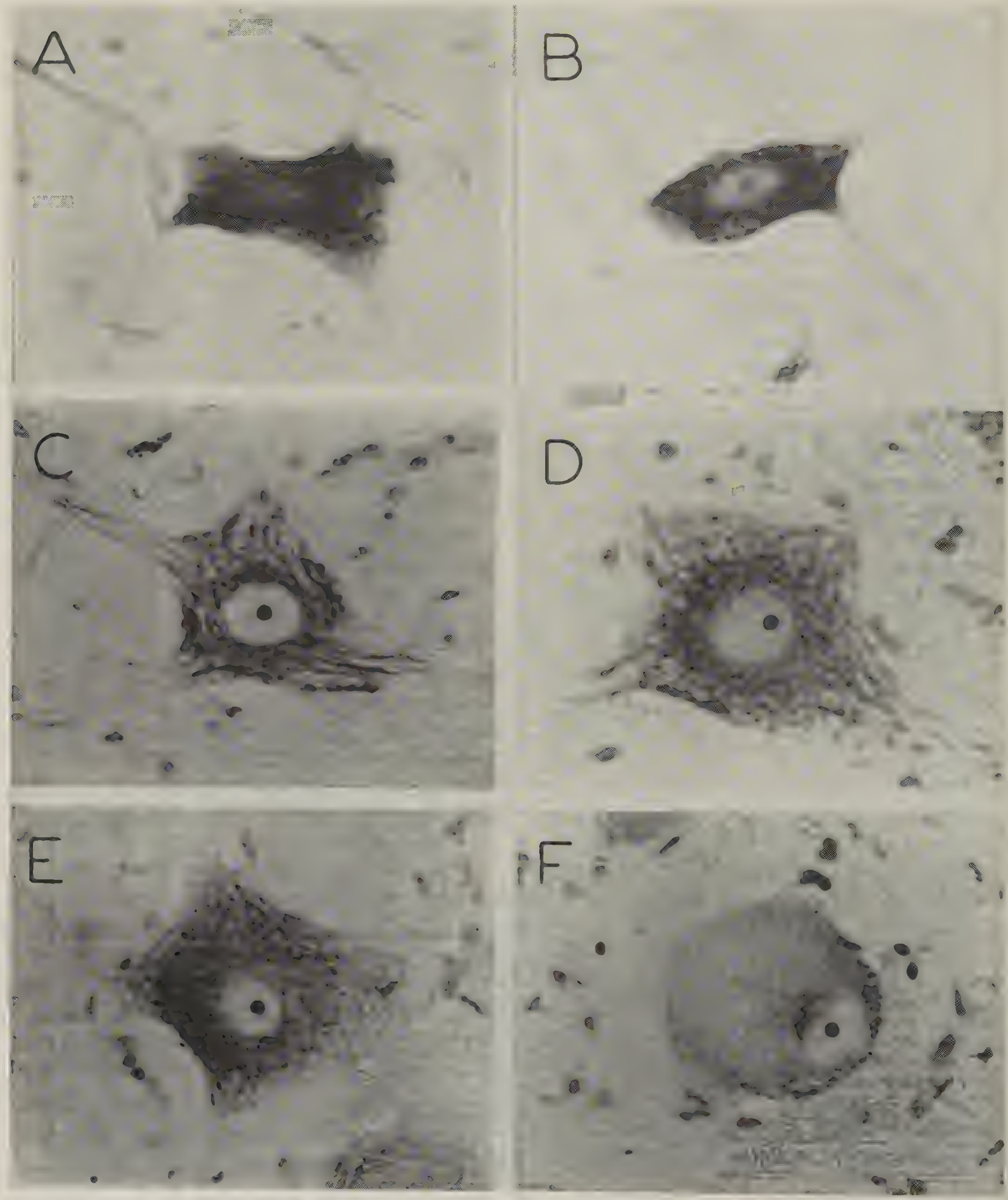

Fig. 1.
Photographs of cells from chromatolytic spinal cords. The designation is that used in Table I × 600.

servations.

In addition to the normal cell, which we have designated N in the accompanying scheme, 3 stages, chosen with regard to the accuracy with which they may be identified, have been differentiated and numbered from 1 to 3. For the sake of completeness, 2 additional categories, A and B, have been set up on the opposite side of the scheme from the chromatolyzed cells to include the

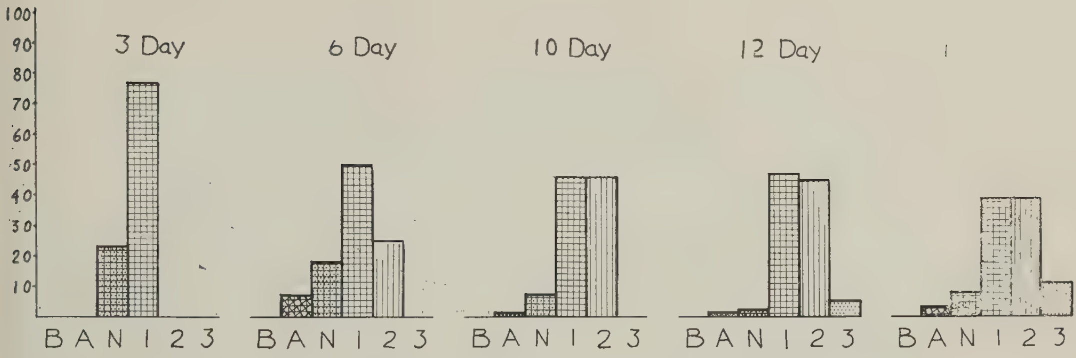

Fig. 2.
Polygon showing the distribution of the differential counts.

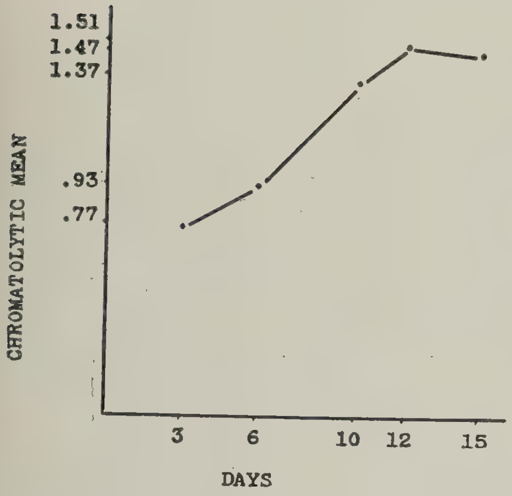

Fig. 3.
Graph showing the relation of the chromatolytic mean index to time.

hyperchromic and the pyknotic cell.

Experiment. Five cats were anaesthetized with pentobarbital and, with sterile operative technic, the first sacral ventral root on the right side of each was sectioned near the spinal ganglion. The animals were allowed to survive for 3, 6, 10, 12, and 15 days. They were killed with pentobarbital and perfused with 10% formalin. The spinal cords were removed, sectioned, stained with cresyl violet and differential counts of the degenerating cells of each preparation were made according to the classification described above.

TABLE II.
Differential Counts of Motor Neurons.

Exp.	Days	B	A	N	1	2	3	Mean
222	3	0	0	23	77	0	0	.77
223	6	0	7	18	50	25	0	.93
224	10	0	1	7	46	46	0	1.37
225	12	0	1	2	47	45	5	1.51
226	15	0	3	8	39	39	11	1.47

The polygons illustrate the sequence of changes which follow root section in these animals. Weighting the columns in Table II by multiplying Col. B by —2, A by —1, N by 0, 1 by 1, etc., allows calculation of the mean for each animal. The relation of the mean to time is shown in Fig. 3.

Summary and Conclusions. The gradual and sometimes subtle differences in cell groups undergoing chromatolysis for varying periods of time may be detected only with a quantitative method. The index presented above serves this purpose and is yet simple and practicable.

15342

Removal of Bone Marrow in Living Animals.

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Experimental suppression of bone marrow activity has been achieved by means of certain chemicals such as benzene and by irradiation. No method has been available for the removal of the entire marrow. The procedure to be described allows the complete extirpation of the whole marrow from the shaft of any of the long bones of a living animal.

Method. The animal of choice is the rabbit. In rabbits along with mice, rats and guinea pigs the marrow in the shaft of the long bones is tubular with relatively few connective tissue bands which binds it to the endosteum. The rabbit is preferable because being the largest of the animals with tubular marrow, the operative procedure involved in the method is technically more accessible. The animal also lends itself readily for hematologic studies.

The animals were anesthetized with pentobarbital sodium. The hair was removed from the extremity with a depilatory preparation and wrapped with cotton saturated with a mild antiseptic. The subsequent surgical procedures were carried out under aseptic precautions to prevent infections of the bone cavity. In the removal of tibial marrow, 2 small incisions are made at each end of the bone. One over the broad anterior surface of the proximal end of the bone and the other over the distal end. A cross incision is made on the broad end to the periosteum and the soft tissue is pulled away. At the narrower end of the bone tendons are retracted. In the case of the femur, humerus, radius and ulna, a single incision from the proximal to the distal ends is preferable. The muscles are separated along the fascial lines and retracted. With a Ralk nail drill a single opening is made at the narrower end through the bone into the marrow cavity. (Fig. 1. 1a). In the wider end, 4 holes are drilled and the bone which is outlined is removed leaving a large opening. (Fig. 1. 1b).

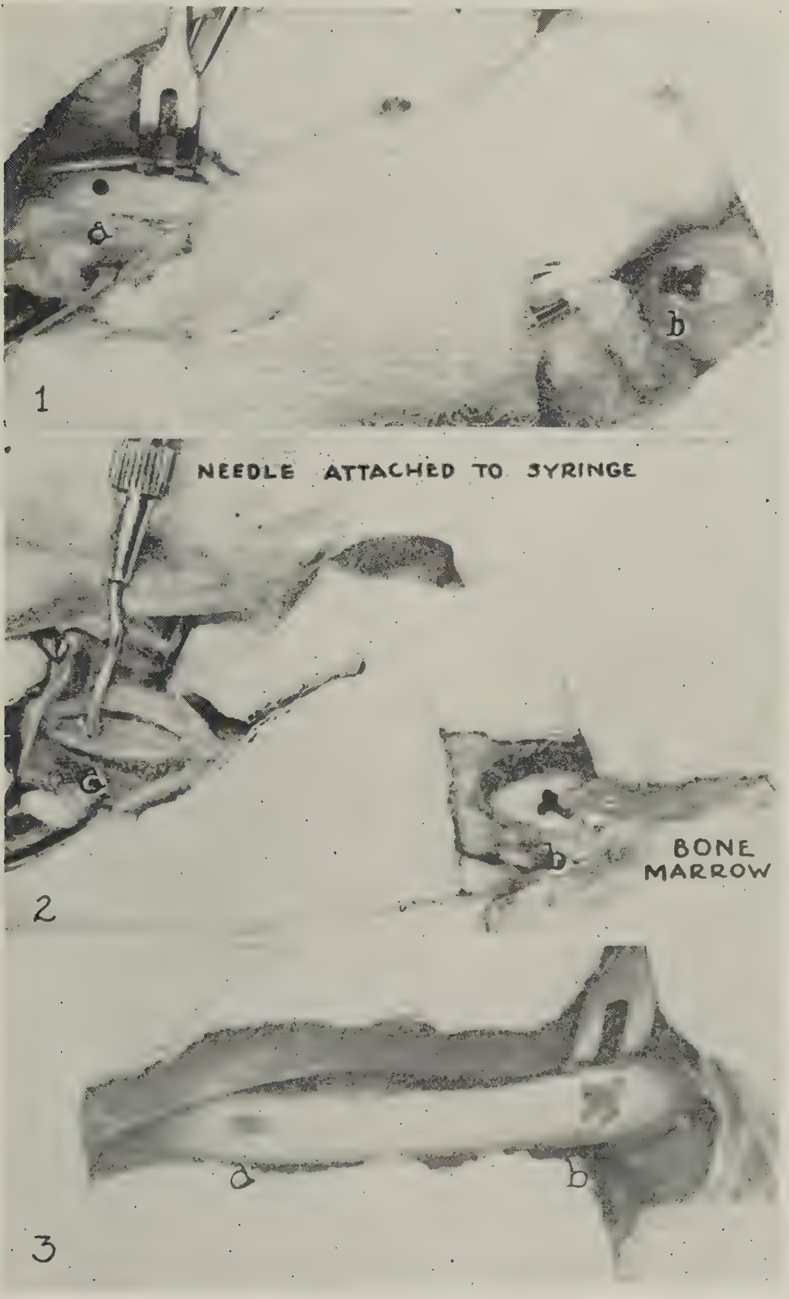
A flexible silver cannula is inserted into the small opening (Fig. 1. 2a). The cannula must fit the opening tightly. A syringe filled with sterile mineral oil is attached to the cannula. The pressure of the oil separates the marrow and expresses it out of the bone cavity through the larger opening (Fig. 1. 2b). The bone cavity is flushed with warm saline. Some marrow remains in the epiphysis of the bone, partly within soft cancellous tissue. If the experiment requires the removal of the entire marrow, the cancellous tissue is first macerated with a fairly rigid metal probe with a hooked tip. The marrow is then washed out with warm saline using the silver cannula and the syringe. There may be severe bleeding from the epiphyses. The bleeding may be controlled by packing both ends with thinly rolled gauze saturated with bone wax. One end of the gauze is left out at the opening. The packing may remain for 10 to 15 minutes or in case of intractable bleeding for 24 to 48 hours at which time it is removed. The openings in the bone are sealed with bone wax. (Fig. 1. 3a,b).

If the tissues are treated with consideration and the muscles are not torn or crushed, the animal shows no ill effects. Mistreatment such as crushing or tearing of tissue may result in shock and death. Undue pressure on the inner structures of the epiphyses predisposes the animal to shock.

The removal of the entire marrow allows the study of the mechanism of whole marrow regeneration and places of origin. The method also presents a testing procedure for the action of substances on regeneration of marrow.

Summary. A method is described allowing the removal of the entire marrow from any of the long bones in a living rabbit.

Fig. 1.
Steps in Removing the Entire Marrow from a Long Bone of a Living Rabbit.



Two holes drilled in the femur with a Ralk nail drill. At one end of the bone a single hole is made (1a). At the opposite end 4 holes are made and the central bone spicule is removed leaving a larger opening (1b). A flexible silver cannula is inserted into the single hole (2a) and with a syringe containing oil or water, the marrow is expressed through the larger opening at (2b). Both openings are then sealed with bone wax (3a and b).

15343

Perosis Caused by Feeding High Levels of Thiouracil.*

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Perosis, or "slipped tendon," is a common deformity of the leg bones of growing chickens, and may be produced by a variety of conditions usually dietary in origin (see review by Ewing).[1] Dietary deficiencies of manganese,[2] choline,[3-5] biotin,[6] nicotinic acid,[7] and probably unidentified factors have been known to produce this abnormality. The condition has been said to be caused also by such factors as excessive minerals in the diet,[8] confinement, wire floors, or it may be inherited.[1,9]

In studies on the effects of thiouracil upon feather pigmentation, it was noted that this drug, when fed at a relatively high level in normal diets, caused the production of perosis in young growing chicks. Results of this and further investigations are presented herein.

Experimental Procedure and Results. Day-old New Hampshire and Barred Rock × New Hampshire crossbred chicks were used. They were raised in electrically-heated batteries with screen bottoms and given the experimental ration and water *ad libitum.* The experiments were conducted for a period of 5 weeks. The thiouracil, which was added to a normal control ration (a typical poultry starting mash),[10] was fed during the entire period.

As may be seen from the summary of results presented in Table I, the addition of 0.5% of thiouracil to the normal control ration resulted in a very high incidence of perosis by the end of the fifth week. The condition was very similar to that ascribed to high levels of minerals in the diet, and to other causes, by the early workers on this problem[9,11,12] and was usually noticeable by the end of the fourth week. The first external symptom noted was a swelling of the tibia-metatarsal joint followed by a shortened appearance and bending of the metatarsus. This was usually accompanied by an outward curling of the toes which has been noted previously in perosis and distinguished from the curled-toe condition of nutritional paralysis.[13] In only about 20% of the chickens was there an actual displacement of the tendon from its condyle.

One group of 10 chicks (group 3) was given, in addition to the thiouracil, a mixture of nutrients known to be necessary to prevent perosis under normal conditions. This was done in an attempt to counteract the effects of thiouracil, if possible. The mixture included choline, nicotinic acid, biotin, and manganese at levels well exceeding ordinary requirements. No improvement was noted.

Because many of the chickens had an outward curling of the toes, riboflavin, known

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* Scientific paper No. A 126. Contribution No. 2014 of the Maryland Agricultural Experiment Station.

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[3] Jukes, T. H., *J. Nutrit.,* 1940, **20**, 445.

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[11] Herner, M. C., and Robinson, A. D., *Poultry Sci.,* 1932, **11**, 283.

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TABLE I.
Perosis Caused by the Feeding of Thiouracil.

Group	Supplement to normal control ration	Total No. chicks started	No. alive at 5 wk	No. with perosis at 5 wk	Avg. weight at 5 wk, g
1	No supplement	41	41	2	345
2	0.5% thiouracil	34	31	30	251
3	0.5% thiouracil, + 0.3% choline, + 10 mg nicotinic acid, + 40 γ biotin, + 100 mg $MnSO_4$ per 100 g of ration	10	10	10	229
4	0.5% thiouracil, + 2 mg riboflavin per 100 g of ration	6	6	6	244

to prevent an inward curling of the toes, was added to the thiouracil ration (group 4). Again, no improvement was noted either in perosis or in the condition of the toes.

In addition to the leg disorder, which caused some difficulty in walking, chickens fed the thiouracil ration showed certain other external symptoms. They grew at a somewhat slower rate than did the control chickens and appeared very fat and stocky. Feather growth was greatly retarded and feathers on the breast and lateral tracts which did appear were very lacy in structure as described by Juhn.[14] In the New Hampshire chickens little, or none, of the usual black pigment appeared in the wing or tail feathers. Comb growth of the males was retarded to such an extent that it was extremely difficult to distinguish the males from the females, even after 6 or 8 weeks. A small percentage of the chickens receiving the thiouracil had excessively scaly feet. Efficiency of feed utilization was similar to the control group.

Autopsies of a few representative thiouracil-treated birds showed the presence of an excessive amount of fat in the abdominal cavity and throughout the body generally, including the muscle tissues. The thyroids were enlarged. These findings are in accord with those of Missouri workers.[15,16]

Discussion. The mechanisms and reactions involved in the formation of perosis are unknown. Wiese, *et al.* reported that there was a decrease in the amount of bone phosphatase activity in chickens deficient in manganese.[17] This was not found to be the case in a choline deficiency.[4] It is probably true that a variety of reactions are involved as evidenced by the number of nutrients necessary to prevent this disorder.

Although no direct evidence is given, the work presented in this study suggests that a lack of thyroxine may also be involved in perosis since it is well accepted that thiouracil depresses the formation of thyroxine (see reviews by Juhn[14] and the Missouri workers).[15,16] If this suggestion is true, perosis should be obtained either by thyroidectomy or by feeding a diet deficient in iodine to chickens. Perosis apparently has not been noted under these conditions.

In this connection, and since a manganese deficiency in the chicken results in perosis, the observation of Reineke and Turner is interesting,[18] that manganese may promote the formation of thyroxine, *in vitro*.

The perosis obtained in this study may be developed independently of the thyroid gland. Thiouracil has been reported to have a direct effect on certain enzymes such as tyrosinase,[19] and on the respiration of bone marrow cells,[20] in addition to its effect on cytochrome oxidase of thyroid tissue.[21]

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[20] Warren, C. O., *Science*, 1945, **102**, 175.

It should be stressed that the level of thiouracil used in this study is higher than that used by most investigations with this drug.

Summary. Perosis, as characterized by an enlargement of the tibia-metatarsal joint and by a thickening and bending of the metatarsal, was obtained in young chicks by the feeding of relatively high levels of thiouracil (0.5%) over a period of 5 weeks. The disorder was not prevented by the addition of manganese, choline, nicotinic acid, biotin, or riboflavin to the thiouracil-containing ration. It is suggested that thyroxine may in some manner aid in the prevention of perosis under normal conditions, although no direct proof for this postulation is offered.

The authors are indebted to Merck and Co., Inc., Rahway, N. J., for the crystalline vitamins; and to Lederle Laboratories, Inc., New York, N. Y., for the thiouracil.

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[21] Paschkis, K. E., Cantarow, A., and Tillson, E. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 148.

15344

Serum Precipitation Reaction in Rheumatic Fever and in Other Conditions.

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Coburn and Pauli[1] described a precipitation reaction which appeared when the serum of a patient in the pre-rheumatic (post-streptococcal) state or Phase II was mixed with the serum of a patient in the acute stage of rheumatic fever or Phase III. The serum in the post-streptococcal period was considered to contain an "antigen" and the serum in the acute stage of rheumatic fever to contain an "antibody." Convalescent serum also contained "antigen." No report confirming or failing to confirm this work has appeared in print. Personal conversation has established, however, that these experiments have been repeated by several workers who have seen the precipitation described, but who could not ascribe any significance to it because of the irregularity of its occurrence.

During 1944-45 through the courtesy and interest of the medical officers* in the Army Air Force stations at Lowry Field, Buckley Field, and Fort Logan, the authors were able to study the serums of some 491 men with diagnoses as follows: 122 rheumatic fever; 181 nasopharyngitis, tonsilitis, and sinusitis; 38 atypical pneumonia; and 150 miscellaneous. The purpose of this paper is to indicate the scope and limitations of the serum precipitation reactions as observed and to suggest a possible relationship to the occasional unfavorable response of patients with acute rheumatic fever to transfusion with blood or plasma.

Terminology. Because of the unknown nature of the substances involved in the reaction, it has seemed desirable to avoid the designations "antigen" and "antibody." Yet to facilitate comparison with the report of Coburn and Pauli, the term "A substance" is used to refer to the "antigen" reactive substance present in the serum prior to the onset of acute rheumatism or during convalescence or a remission, and the term "B substance" is used to refer to the "antibody" reactive substance present during active rheumatism. (It is likely that these terms

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‡ Present address: Camp Detrick, Frederick, Md.

[1] Coburn, A. F., and Pauli, Ruth H., *J. Exp. Med.*, 1939, **69**, 143.

* Especially Lt. Col. L. D. Williams, Capt. C. L. Hoff, Capt. G. L. Erdman, Major O. G. Wilson, Lt. Col. W. B. Adamson, Capt. G. Blankfort, Major F. Foster, Major L. R. Grams.

TABLE I.
Cross Reactions Between 188 Serums Which Gave at Least One 2-Plus (++) or Stronger Precipitate.

Nature of serums used					
Serum 1	Serum 2	No. of tests	Reaction ++ or more	Reaction + to ?	Reaction 0
A	B	438	235	114	89
A	AB	69	43	17	9
B	AB	57	29	12	16
A	A	148	1	3	144
B	B	88	0	29	59
AB	AB	3	1	2	0
Totals		803	309	177	317

should be reversed if antigen or antibody is actually implicated).

Methods. We have found that a study of these phase substances was facilitated by bleeding several patients during the first 3 days of acute rheumatic fever and other patients in the 10-20th day of the disease and testing all serums so obtained against each other. The serums fell into 4 groups (1) A, (2) B, (3) A and B mixed, (4) negative. Further serum specimens were then classified by testing against one or more A serums and against one or more B serums.

Blood serum was preserved with merthiolate in a final concentration of approximately 1-10,000 and stored at 5°C. Centrifugation prior to use was sometimes necessary to clear. About 0.1 cc of A serum was mixed by shaking with 0.1 cc B serum in a chemically clean precipitin tube, which had an inner diameter of 2 mm, incubated in a water bath at 41°C 2 hours, and the readings made with the aid of a dualoupe binocular 3 × magnifier. The tubes were then placed in the refrigerator overnight, allowed to warm to room temperature and read again. The serums should not be allowed to freeze. The suspended precipitate after overnight refrigeration was sometimes stronger, but more anomalous effects appeared than if the reading was made on a basis of the results after 2 hours at 41°C. The observed reaction was in the form of a suspended precipitate which looked much like a positive Kahn test. The intensity was read as +++ flakes, ++ large granules, + small granules, ± very fine granules, 0 clear. Miss Pauli viewed some of the readings and found them to correspond with those reported in 1939. It is important that every serum be set up by itself as a control because some serums precipitate spontaneously. These were thought to contain a mixture of A substance and B substance. Such serums often contained predominantly one or the other. As a serum became over 2 months old there was an increasing tendency for it to precipitate by itself or for it to lose potency even though it did not become self-precipitating.

Practically all tests were with heterologous serums. Only a small series was with homologous serums, sufficient to confirm the fact that A serum would react with B serum from the same patient.

The most difficult aspect was to secure and maintain a supply of serums containing dependable A and B substances. In civilian life this is accentuated by the small amounts of blood available from children. No sooner is a serum well established as having a dependable A or B content which could be used to classify other serums than it is exhausted.

Cross Reactions Between Serums. Table I summarizes the results of 803 tests on 188 serums from 123 patients. Of the 188 serums, 115 were predominantly A serums, 59 were B serums, and 14 were mixed. Of the 123 patients, 51 had rheumatic fever, 40 atypical pneumonia, 23 nasopharyngitis, and 9 miscellaneous conditions, including 2 with mumps.

This table includes all the serums which gave at least one 2 plus reaction. Only the maximum reaction between 2 serums is recorded, in case 2 serums were tested against one another more than once.

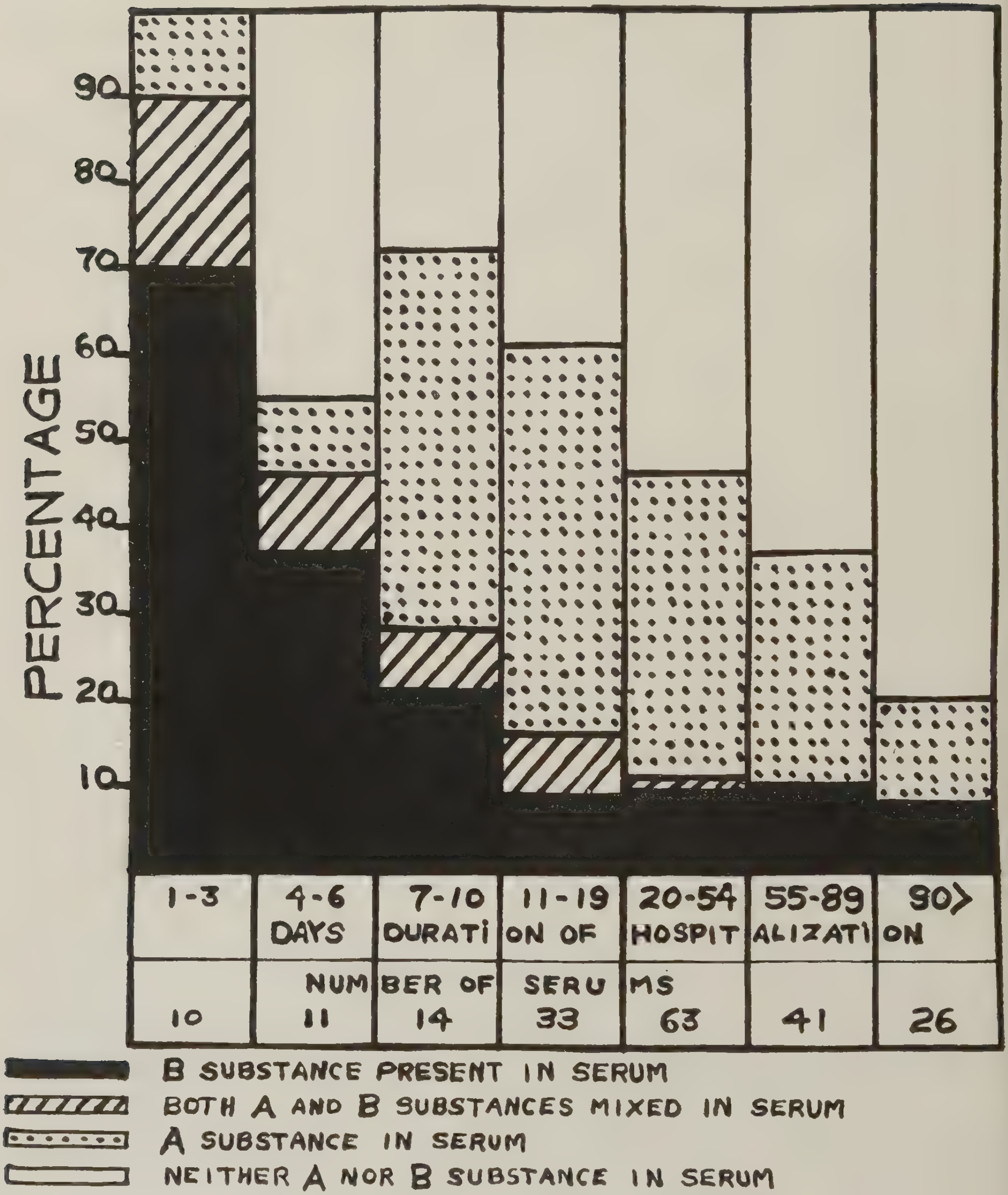

Fig. 1.

Distribution of A and B substances in 98 patients with rheumatic fever in relation to duration of hospitalization.

Table I shows that in 438 tests of an A serum against a B serum there were 2 plus reactions in 235 tests and no reactions in 89 tests, which suggests a quantitative (possibly also qualitative) difference in amount of A or B substance present in any serum. When 88 tests were made by combining one B serum with another, there were 29 weak reactions and 59 negatives. This suggests that the B serums had only a predominant content of this substance but that they did contain some small amount of A substance also.

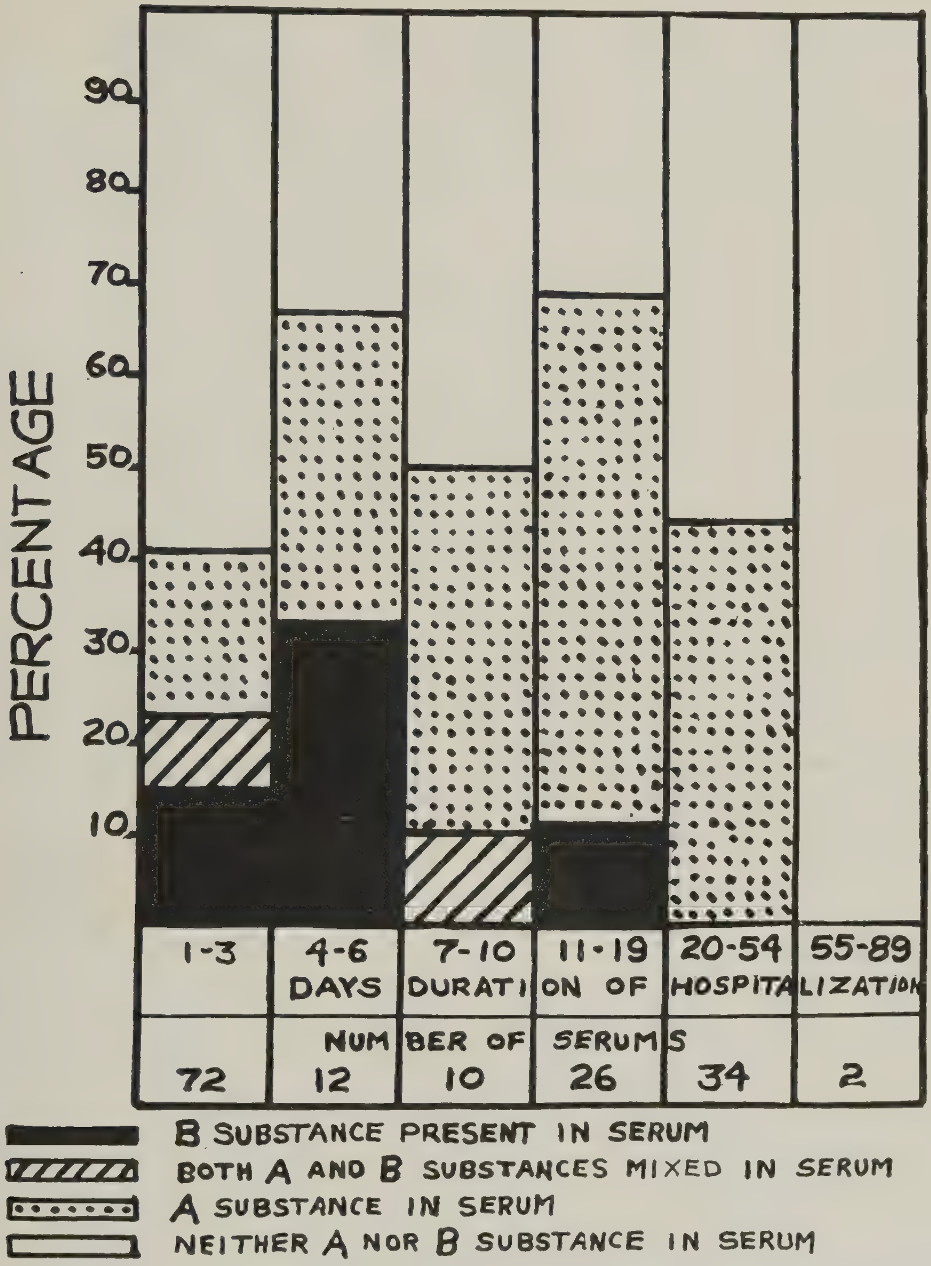

Fig. 2.

Distribution of A and B substances in 116 patients with nasopharyngitis in relation to duration of hospitalization.

Serums from patients with rheumatic fever, atypical pneumonia, and nasopharyngitis cross-reacted; hence the phenomenon was not exclusively connected with any specific disease.

A and B Substances in Relation to Duration of Disease. Fig. 1 presents the results obtained with 198 serums from 98 patients with rheumatic fever, diagramed to show whether the patients were in an A or B phase at the time the blood was drawn. No patient appears in any one category more than once.

The first day of disease was considered to be the first day of hospitalization. This is a reasonably accurate assumption for the military men concerned because hospitalization usually occurred early.

It can be seen that during the first 3 days of hospitalization 7 of 10 patients were in the B phase but that after the 10th day only 15 of 163 sera contained this substance predominantly. Those which persisted in a B phase after the 40th day were principally instances of recurrences of rheumatic fever or prolonged active rheumatic fever. The number in the A phase was low at first, increased until about the 20th day, and then declined while those with negative serums increased. This same general tendency was also present in 87 serums from 34 patients with atypical pneumonia and in 156 serums from 116 patients with nasopharyngitis, but in both instances there were more serums which contained neither A nor B substance. This is illustrated for nasopharyngitis in Fig. 2. Serums from patients with rheumatic fever contained A or B substances more frequently than did those from other conditions studied by us.

Sequence in the Appearance of B and A Substances. There was a definite order of appearance of B and A as follows: (1) acute illness-B phase, (2) subsidence of illness-mixed B and A, (3) convalescence-A phase, (4) recovery-negative phase, (5) recurrence of illness-B phase. This is illustrated in Fig. 3 which shows 85 serums from 28 patients whose diagnoses were as follows: 14 rheumatic fever, 7 nasopharyngitis, 5 atypical pneumonia, and 2 nasopharyngitis with later rheumatic fever. Multiple specimens of serum were taken from each patient. The time intervals varied, but each patient was in the B phase when first bled. Presence of B phase in the 4th, 5th and 6th bleedings was found in 2 patients with prolonged active rheumatic fever. Evidence of the above sequence was also seen in 10 patients whose first serum contained mixed A and B, and in 32 who were in the A phase when first bled. In 41 patients initially negative, a number went into the A phase without passing through an observed B phase.

In all these observations consideration must be given to the variable titer of the serums and the irregularity of the times at which patients were bled, which caused difficulty in classification of serums and detection of the sequence of phase substances.

The following case histories will illustrate the sequence of events:

Hospital day	Case 3
1	Scarlet fever
14	Acute R. F. with polyarthritis
61	O phase substance in serum
77	Recurrence of acute R. F.
81	Only B substance in serum
93	Only A substance in serum
123	O phase substance in serum
124	Returned to active duty
	Case 26
1	Atypical pneumonia
3	X-ray L. L. L. atypical pneumonia
12	X-ray resolved atypical pneumonia
14	Only A substance in serum
18	X-ray recurrence atypical pneumonia
20	Only B substance in serum
26	X-ray resolved atypical pneumonia
41	Only A substance in serum
57	Returned to active duty
84	O phase substance in serum (on duty); no prior history of R. F.; no arthritis or carditis during hospitalization
	Case 62
1	Acute R. F. with polyarthritis
4	Only B substance in serum
10	Only B substance in serum
36	Only A substance in serum
66	Only A substance in serum
97	O phase substance in serum
100	Sent to Arizona as convalescent R. F.
	Case 123
1	Nasopharyngitis
2	Mixed A and B substances in serum
4	Only A substance in serum
7	Returned to active duty
48	O phase substance (on duty)

Phase Substance in Relation to Transfusions. In patients with rheumatic fever partial collapse has been reported after the administration of convalescent serum[2] and recurrence of rheumatic fever after administration of serum obtained during the acute phase of the disease in the donor.[3] Blood plasma from normal donors caused an increase in rheumatic activity in 7 of 9 patients.[4] At

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[2] Green, C. A., Glazebrook, A. J., Thompson, S., and Hopkins, W. A., *Proc. Roy. Soc. Med.*, 1940, **33**, 275.

[3] Friedman, M., Klein, R., and Rosenblum, P.,

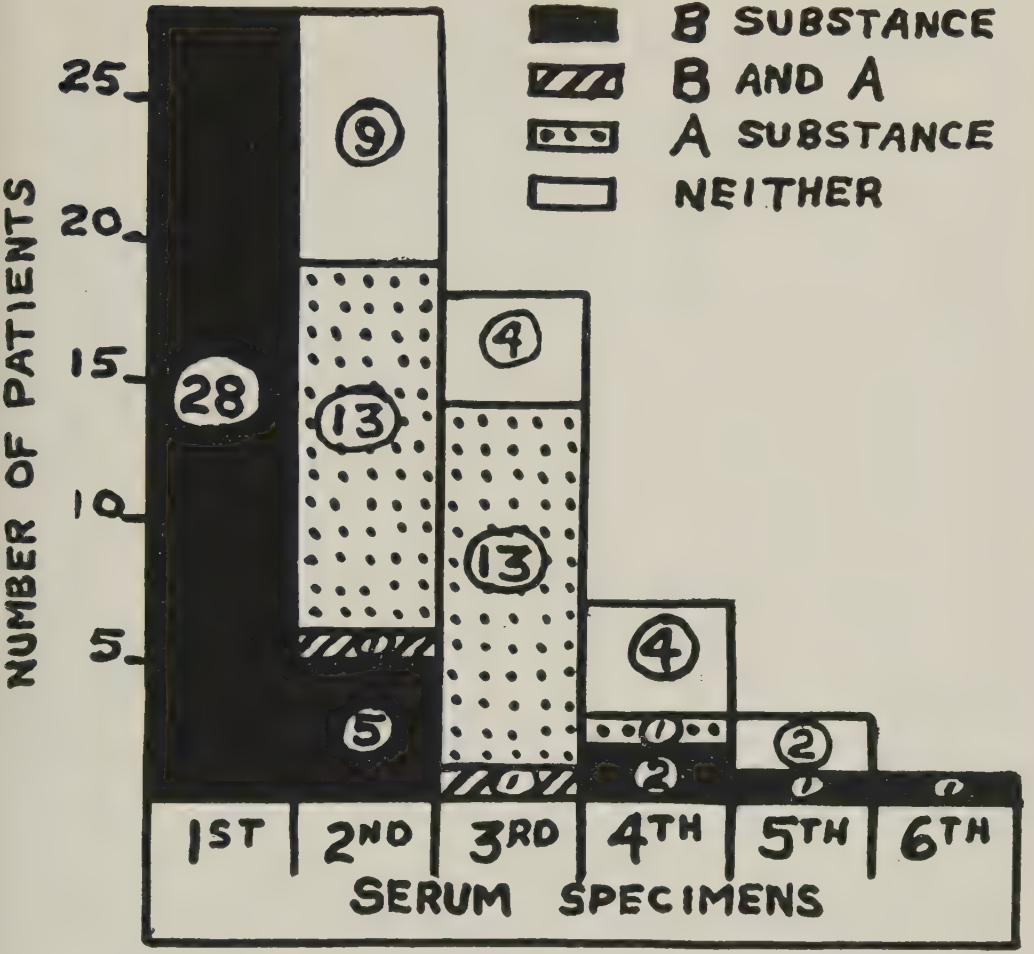

Fig. 3.
Sequence of B and A substances in 28 patients yielding multiple serum specimens at irregular time intervals during and after hospitalization.

Lowry Field, 2 patients had an unfavorable clinical response to compatible blood transfusions. It may be significant that in each case the recipients were definitely in B phase and the 5 donors were in an A phase, so that when the serums of recipient and donor were mixed, a suspended precipitate formed. Among 64 recipients of transfusions at Colorado General Hospital, the B substance was present in 16 patients, in such diverse conditions as Hodgkins disease, active tuberculosis with abortion, lung abscess with surgical drainage, carcinoma of the stomach, burns, abscess posterior to the stomach from perforated peptic ulcer, arteriosclerotic gangrene with amputation. Among 38 civilian donors there was a questionable A phase in only 2 and no B phase. Study of this "phase reaction" in a large transfusion service might yield interesting results.

Other Experiments. In a few trials, heating serums to 55°C for 30 minutes decreased their ability to react. Complement had no restorative effect. In one serum use of a fritted glass filter quantitatively reduced the potency of the A substance, but Berkefeld filtration did not appreciably affect either A

Am. J. Dis. Child., 1938, **56**, 1304.

[4] Griffith, G. C., Leake, W. H., and Butt, Hugh, *Modern Concepts of Cardio-vascular Disease*, published monthly by the American Heart Assn., 1945, **14**, 7.

or B substances in 3 instances. No absolute correlation was observed between presence or absence of A or B substances and the blood group of the donors,† blood sedimentation rate,† serum antistreptolysin titer,† warm or cold agglutinins for red blood cells, sheep red cell heterophile antibody titer,† or the presence of hemolytic streptococci in the throat.†

Comment. A clear serum tended to precipitate with a turbid (even after centrifugation or filtration) serum but there was no uniform relationship. Two clear serums or 2 turbid serums might react. Possibly the turbid serum merely furnished a suitable colloidal state similar to that when collodion particles are used to detect small quantities of antibody.

Whether the reactions observed are true immunological phenomena or only aspects of serum lability is not clear. The similarity to the precipitinogen in yellow fever as described by Hughes[5] and Lennette[6] is noteworthy. There have been reports which suggest the existence of similar reactive substances in typhus[7] post-vaccinal hepatitis,[8] and infectious myxomatosis of rabbits.[9] There may exist a whole series of closely related auto-antigens which vary slightly with each disease.

Close examination of a recent paper[10] on electrophoretic changes in the serum protein patterns of patients with scarlet fever and rheumatic fever reveals that the maximum reduction (value 0.7) in the albumin-globulin ratio in the 3 patients with rheumatic fever occurred at the onset of joint pains. This is the point at which we would be most likely to find "B substance" in the serum. Similarly the only patient with scarlet fever who developed a similar reduction of the albumin-globulin ratio was one whose course was complicated by purulent sinusitis. He also would, in our experience, be more likely to have "B substance" than those patients with uncomplicated scarlet fever. The question is then whether the B phase represents a serum lability caused by the lowered albumin-globulin ratio.

Summary. 1. When the serum of a patient in the early acute stage of rheumatic fever was mixed and incubated with a convalescent serum taken at an appropriate time from himself or another rheumatic patient, a precipitate formed. 2. The "phase substances" causing this reaction were present, but less frequently, in atypical pneumonia, nasopharyngitis, several other diseases, and rarely, in apparently normal blood donors. 3. Because cross-reactions occurred between serums from patients with different diseases, it is difficult to ascribe any etiological significance to the findings. 4. This reaction may be responsible for the occasional unfavorable response to blood or plasma transfusion of patients with rheumatic fever. 5. It is not clear whether this phenomenon is a manifestation of serum lability or of the formation of an auto-antigen-antibody system.

The authors wish to thank Mrs. Ella Jane Swan for her voluntary technical assistance and Dr. Homer F. Swift for his criticism and comments.

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† These procedures were performed by the A. A. F. laboratories at Lowry and Buckley Fields.

[5] Hughes, T. P., *J. Immunol.*, 1933, **25**, 275,

[6] Lennette, E. H., and Perlowagora, Alina, *Am. J. Trop. Med.*, 1945, **25**, 11.

[7] Smorodintzeff, A. A., and Fradkina, R. V., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 93.

[8] Sawyer, W. A., Meyer, K. F., Eaton, M. D., Bauer, J. H., Putman, P., and Schwentker, F. F., *Am. J. Hyg.*, 1944, **40**, 35.

[9] Rivers, T. M., Ward, S. M., and Smadel, J. E., *J. Exp. Med.*, 1939, **69**, 31.

[10] Dole, V. P., Watson, R. F., Rothbard, S., Braun, E., and Winfield, K., *J. Clin. Invest.*, 1945, **24**, 648.

15345

The Antagonistic Action of Choline and its Triethyl Analogue.*

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In recent years, considerable interest has been developed in the interference with biological processes through the use of analogues of essential metabolites.

Triethyl choline chloride, the ethyl analogue of choline, is an interesting compound from this point of view. Triethyl choline is not capable of replacing choline in biological processes in which choline acts as a methyl donor,[1] but its lipotropic activity has been found to be substantially equal to that of choline itself.[2] Since the formation of acetyl choline presumably would not involve transmethylation directly, the triethyl derivative might possibly be handled similarly to choline in the systems involving its acetylation. The acetyl derivative of triethyl choline, however, does not have any of the stimulating actions of acetyl choline. A competition with choline in these systems should therefore lead to diminished formation of active acetyl derivatives.

Triethyl choline chloride, when injected into animals in sufficient amounts, is acutely toxic.[3] The symptoms demonstrated include apparent muscular weakness, apparent exophthalmos, and convulsions, followed by death within a few minutes. On the assumption that this acute toxicity is due to competition with choline in an essential biological system, choline was administered to see whether it would protect animals against its triethyl analogue.

Forty-four mice, ranging in weight from 16 to 23 g, were divided into 2 groups containing animals of corresponding weights. The mice of one group were injected in the hind leg with triethyl choline chloride, prepared according to the directions of Channon and Smith.[4] Six out of 9 mice given 1.25 mg died. Twelve out of 13 mice given 1.75 mg died.

The mice of the other group were treated with the same amounts of triethyl choline chloride and an equal weight of choline chloride. Out of 9 mice given 1.25 mg triethyl choline chloride together with 1.25 mg choline chloride, all survived. Out of 13 mice given 1.75 mg triethyl choline chloride together with 1.75 mg choline chloride, 12 survived.

In order to investigate further the mechanism of the toxicity of the compound, the contraction of isolated frog muscles (gastrocnemius and rectus abdominus) was studied in the presence and absence of the triethyl derivative. The muscle was suspended from a muscle lever in the usual way in frog Ringers solution and the contractions recorded with a kymograph. When triethyl choline was placed in the solution bathing the muscle, it very substantially blocked the response of the muscle to choline, while it did not appreciably block the response to acetyl choline. On washing the muscle, the response to choline was very largely restored. When muscles were contracted with choline, addition of the triethyl derivative to the fluid containing the choline, resulted in a rapid relaxation of the muscles. No such effect was observed when acetyl choline was employed. Table I is a summary of a typical experiment.

These findings are consistent with the assumption that the compound interferes with acetyl choline formation, if choline does not act directly on nerve-muscle preparations but acts by forming or making available acetyl choline. Aside from the fact that choline

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* Aided by a grant from the John and Mary Markle Foundation.

[1] Moyer and du Vigneaud, *J. Biol. Chem.*, 1942, **143**, 373.

[2] Channon, Platt, and Smith, *Biochem. J.*, 1937, **31**, 1736.

[3] Hunt and Taveau, *J. Pharm. and Exp. Therap.*, 1909-10, **1**, 303.

[4] Channon and Smith, *Biochem. J.*, 1936, **30**, 115.

TABLE I.

Muscle: Frog gastrocnemius
Lever arm: 1:12

Time	Substance added	Concentration mg per ml	Contraction in mm
1:24	acetyl choline chloride	0.015	28
:28	wash 3 times		
:30	acetyl choline chloride	0.015	28
:34	wash 3 times		
:45	choline chloride	1.3	26
:50	wash 3 times		
2:00	triethyl choline chloride	1.3	0
:15	choline chloride	1.3	3
:20	wash 3 times		
:23	choline chloride	1.3	19
:31	wash 3 times		
:58	acetyl choline chloride	0.015	29
3:01	wash 3 times		
:20	acetyl choline chloride	0.015	27
:24	wash 3 times		
:26	triethyl choline chloride	1.3	0
:40	acetyl choline chloride	0.015	29

is a presumable precursor of acetyl choline, choline may act to liberate acetyl choline by cationic exchange adsorption, as suggested for the mode of action of choline derivatives by Renshaw, Ziff and Green.[5]

An interesting aspect of triethyl choline chloride is that it represents a compound which can replace a metabolite in one process but is antagonistic to it in another physiological function.

The authors are indebted to Miss Edith Ludmer for her assistance in these experiments.

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[5] Renshaw, Ziff, and Green, *J. Pharm. and Exp. Therap.*, 1938, **62**, 430.

15346 P

Anticoagulant Activity of the Trypsin Inhibitor from Soya Bean Flour.

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We have observed that the trypsin inhibitor from unheated soya bean flour[1,2] has marked anticoagulant activity on human whole blood, recalcified plasma and recalcified plasma to which an excess of thromboplastin is added. (Table I). However, the soya bean inhibitor has no antithrombic activity. We have also observed that this material inhibits the proteolytic enzyme of blood plasma,[3] which may play a role in the coagulation of blood. This is true whether the plasma enzyme has been activated by chloroform treatment or by the addition of streptolysin.[4,5]

A crude preparation of inhibitor was obtained by the method of Ham *et al.*[1] An excess of potassium oxalate was added to the acetone free material in order to precipitate the calcium. The solution was dialyzed 48 hours against running tap water. It was rendered isotonic by addition of NaCl and

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[1] Ham, W. E., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, **161**, 635.

[2] Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

[3] Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 525.

[4] Christensen, L. R., *J. Bacteriology*, 1944, **47**, 471.

[5] Kaplan, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 40.

TABLE I.
Anticoagulant Activity of Trypsin Inhibitor from Soya Bean Flour.

	Reagents						
Crystalline inhibitor (mg)	Whole blood (cc)	Citrated plasma (cc)	$CaCl_2$ (0.25%) (cc)	Fibrinogen (cc)	Thromboplastin (cc)	Thrombin (cc)	Clotting time
*0	2						8 min.
0.2	2						18 ”
†0		0.1	0.15				6 ”
†0.3		0.1	0.15				21 ”
0		0.1	0.15		0.1		13 sec.
0.1		0.1	0.15		0.1		20 ”
†0				0.1		0.1	10 ”
†0.5				0.1		0.1	10 ”

* 0.2 cc of saline added.
† Total volume of 1 cc in test tube, by addition of imidazole buffer at pH 7.4.

brought to pH 7.4 by NaOH N/10. The results obtained with the crude material were confirmed with a purified preparation 4 times recrystallized,[2] secured through the courtesy of Dr. Moses Kunitz.

All experiments were conducted at pH 7.2-7.4 on human blood, citrated plasma and fibrinogen, in imidazole buffer, at 37°C. The streptolysin was a dry powder, dissolved in saline.*,[5] Control experiments showed that the anticlotting and antiproteolytic activity were not due to the small amount of salt present in the crystalline preparation and that the addition of an excess of calcium did not modify the anticlotting activity. Autoclaving of the crude material at 120°C for 20 minutes completely abolished the anticoagulant activity and almost completely the antiproteolytic activity.

These facts represent a new instance of the inhibition of the clotting of blood by trypsin inhibitors.[6,7]

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* Supplied by the Commission on Acute Respiratory Diseases, through the courtesy of M. H. Kaplan.

[6] Grob, D., *J. Gen. Physiol.*, 1943, **26**, 423.

[7] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 373.

15347

Protective Effect of Glutathione and Cysteine Against Alloxan Diabetes in the Rat.*

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Although Wiener[1] reported that alloxan produces fatal convulsions in rabbits, the mechanism of death was not understood until Jacobs[2] demonstrated that injections of alloxan into rabbits resulted in fatal hypoglycemia. It only became apparent that alloxan could produce experimental diabetes when Dunn, Sheehan, and McLetchie[3] reported that alloxan produces necrosis of the beta cells of the islets of Langerhans. Many workers have since confirmed these observations.

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* Presented, in part, at a sectional meeting of the PROC. SOC. EXP. BIOL. AND MED., Cleveland, Ohio, April 13, 1945.

[1] Wiener, H., *Arch. f. exp. Path. u. Pharm.*, 1899, **42**, 375.

[2] Jacobs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 404.

[3] Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet*, 1943, **1**, 484.

The mechanism of alloxan action is not understood. Since alloxan combines with sulfhydryl groups of protein[4] and since many enzymes require sulfhydryl groups for their activity,[5,6,7] it seemed possible that necrosis might result from enzyme inactivation by alloxan. Thus, it is noted that alloxan inhibits the activity of succinic dehydrogenase[5] as well as the proteolytic enzymes, papain and cathepsin.[6] Active sulfhydryl groups are also necessary for pyruvate oxidation and condensation, malate and ketogluterate oxidation, d-amino acid and l-glutamic acid oxidation, and various fatty acid oxidations.[7]

Because many of the enzymes inactivated by alloxan can be reactivated, in part at least, by adding glutathione,[5,7] a naturally occurring sulfhydryl compound, it was decided to test the effect of glutathione on the course of alloxan diabetes. Cysteine was used for part of the study because it is much cheaper than glutathione and because the sulfhydryl group of glutathione is contained in the cysteine component of the molecule.

In a preliminary note[8] I reported that the injection of alloxan (200 mg/kg) intraperitoneally into rats immediately following an intravenous dose of neutralized cysteine or glutathione (7.5 mM/kg) did not cause diabetes. The injection of alanine, glycine, phosphate buffer, ascorbic acid, or succinic acid in place of the cysteine or glutathione did not protect the animals against alloxan. Leech and Bailey[9] reported that the injection of 300-400 mg/kg of glutathione simultaneously with alloxan did not protect rabbits from diabetes, and Weinglass *et al.*[10] reported that giving 300 mg/kg of alloxan subcutaneously simultaneously with 500 mg/kg of cysteine-HCl did not afford protection to rats. However, it is to be noted that the doses of cysteine and glutathione which I used were much greater than those used by other investigators, for 7.5 mM/kg is equivalent to 912 mg/kg of cysteine and 2,500 mg/kg of glutathione.

Methods. In the preliminary studies alloxan was injected intraperitoneally. The intraperitoneal injection of alloxan into rats in doses of 200 mg/kg was not found to be reliable, for, although this dose usually produced diabetes in about 80% of the rats, occasionally an entire group of animals failed to respond. In contrast, intravenous injections of alloxan in doses of 40 mg/kg produced diabetes in 95% of the animals.[11] Therefore, this dose and route of administration were used in the present studies.

To facilitate the intravenous injection, vasodilation was induced by placing the rats in a 56°C oven for several minutes. A 2% solution of alloxan monohydrate was injected into the tail vein and blood was always drawn into the syringe before the injection was started. Solutions of cysteine hydrochloride or glutathione were carefully neutralized to pH 7.4 with NaOH and diluted so that each cc contained 0.2 to 0.75 mM. Usually one cc of solution was injected per 100 g of body weight. The cysteine was given either before or after the alloxan and different veins were used for each injection. As controls the following compounds were used and were injected in place of cysteine or glutathione:

1. NaCl (7.5 mM/kg), because NaCl is formed in the neutralization of cysteine-HCl.
2. Phosphate buffer, pH 7.4 (2.5 mM/kg), to rule out the effect of pH. Doses larger than 2.5 mM/kg caused a convulsive death, apparently due to hypocalcemic tetany.
3. Alanine, to test whether the protective

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[4] Lieben, F., and Edel, E., *Biochem. Z.*, 1933, **259**, 8.

[5] Hopkins, F. G., Morgan, E. S., and Lutwak-Mann, C., *Biochem. J.*, 1938, **32**, 1829.

[6] Purr, A., *Biochem. J.*, 1935, **29**, 13.

[7] Barron, E. S. G., and Singer, T. P., *J. Biol. Chem.*, 1945, **157**, 221; Singer, T. P., and Barron, E. S. G., *J. Biol. Chem.*, 1945, **157**, 241.

[8] Lazarow, A., *Anat. Rec.*, 1945, **91**, 24.

[9] Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, 1945, **157**, 525.

[10] Weinglass, A. R., Frame, E. G., and Williams, R. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 216.

[11] Lazarow, A., and Palay, S. L., in press, 1946.

TABLE I.
Protection Against Alloxan Diabetes. Protective Agent Given Intravenously 1-2 Minutes Before Alloxan. Alloxan 40 mg/kg Intravenously, Given Immediately After Protective Agent.

Protective Agent	Dose in mM/kg	No. rats	% diabetic	Avg. 48-hour blood sugar	
				Diabetic	Non-diabetic
None	—	19	95	397	—
Cysteine	7.5	13	0	—	130
"	5.0	2	0	—	108
"	2.5	2	0	—	105
"	1.0	8	12	318	142
"	0.5	6	66	301	138
"	0.33	2	100	296	—
Glutathione	2.0	5	0	—	108
"	1.0	5	60	345	122
Thioglycolic acid	7.5	4	0	—	155
Alanine	7.5	4	100	368	—
NaCl	7.5	4	100	477	—
Phosphate buff. pH 7.4	2.5	5	100	467	—
Ascorbic acid	4.0-7.5	4	100	610	—

effect of cysteine was due to the amino acid or the SH group.

4. Ascorbic acid neutralized to pH 7.4 in doses of 4.0-7.5 mM/kg, in order to test the specificity of the SH reducing groups, as ascorbic acid is also a reducing agent.

5. Thioglycolic acid, another sulfhydryl compound, was also used.

The rats (Sprague-Dawley) were allowed excess food and water throughout the experiment. Blood sugars were determined by the Folin-Malmros micro method[12] on blood obtained by cutting the tip of the tail. Serial sugars were determined at 0, 3, 8, 24, 48 and, occasionally, at 72 hours. The rats were usually killed at 48 hours although a few were killed at 72 hours.

Data. Following the injection of alloxan 3 phases of the blood sugar response are noted—an initial hyperglycemia, followed by a period of hypoglycemia, and finally, a late stage of permanent hyperglycemia or diabetes. As has been previously reported[11] animals injected intravenously with 40 mg/kg of alloxan usually reached their permanent hyperglycemic phase by 48 hours. Although, very occasionally, a late hypoglycemia occurred at 48 hours, these animals were also considered diabetic, for histologically the beta cells showed extensive necrosis. Animals were considered normal when the blood sugars at 48 hours were normal and all of the serial blood sugars were within the range of 90-160 mg %. In rats which were partially protected from the action of alloxan by inadequate doses of cysteine, slight initial hyperglycemic and hypoglycemic reactions were noted. The 48-hour sugars were normal or only slightly elevated but at 72 hours the sugars did not exceed 250 mg %. These animals were classed as mild diabetics. The tables list the average 48-hour blood sugars for the diabetic and normal animals and the 72-hour blood sugars for the mild diabetic animals. The biochemical findings presented in this paper correlate well with the cytological findings in the pancreas, which are reported in another paper.[13]

Reference to Table I shows that the intravenous injection of cysteine in doses of 2 mM/kg or greater, immediately before the administration of 40 mg of alloxan (equivalent to .25 mM/kg of alloxan) completely protected these animals against experimental diabetes. Cysteine dosages of 1 mM/kg protected most of the animals whereas with smaller doses nearly all the animals developed diabetes. When glutathione was used, complete protection was obtained when the intravenous dose was 2 mM/kg, but when glutathione was given in doses of 1 mM/kg

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[12] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

[13] Palay, S. L., and Lazarow, A., in press, 1946.

TABLE II.
Protection Against Alloxan Diabetes. Cysteine 7.5 mM/kg Given Intravenously Either Before or After Alloxan 40 mg/kg I. V.

Substance given first	Time interval min.	No. animals	% diabetic		Blood sugars			
			Severe	Mild	48-hr severe diabetic	48-hr mild diabetic	72-hr mild diabetic	48-hr non-diabetic
Cysteine	1-2	13	0	0	—	—	—	130
Alloxan	15	5	80	0	471	—	—	91
,,	6	6	83	0	503	—	—	112
,,	3	3	66	33	454	173	300	—
,,	1	10	10	60	390	133	214	140

only some of the animals were protected. Thioglycolic acid in doses of 7.5 mM/kg was the only other agent tested which had any protective effect. Alanine, sodium chloride, phosphate buffer or ascorbic acid did not modify the course of alloxan diabetes.

Reference to Table II shows that when the alloxan was given prior to the sulfhydryl compound some protection against diabetes resulted if the time interval between the alloxan and the cysteine injections was small. If the interval was one minute, only one of 10 animals developed severe diabetes and 5 showed mild diabetes. Cytologically, however, all of these animals showed some evidence of pancreatic damage, although in 9 out of the 10 rats this consisted only of degranulation or necrosis of some of the beta cells in the islets. However, if the time interval between alloxan and cysteine was 3 minutes or longer, there was no significant protection against diabetes.

All of the cysteine-protected animals in this group were killed at the end of 48 or 72 hours for histological examination so that follow-up studies were not made. However, some of the rats reported in the preliminary note[8] were followed for several months. These had received 7.5 mM/kg of cysteine intravenously immediately preceding 200 mg/kg of alloxan intraperitoneally. None ever did develop diabetes.†

Discussion. At pH 7.4 *in vitro* alloxan is rapidly converted to a non-diabetogenic compound, alloxanic acid.[14] The fact that large doses of phosphate buffer (pH 7.4) injected intravenously just prior to the injection of alloxan did not protect animals against diabetes indicated that the cysteine effect is not due to any contained buffer. The fact that alanine did not protect against alloxan diabetes, whereas cysteine did, indicates that the protection is not due to a direct interaction between alloxan and amino acids—a reaction which is known to occur in the test tube.[15] Since thioglycolic acid also protected against diabetes, the SH group appears to be responsible for the protective action of cysteine and glutathione.

Since the liver controls the addition of sugar to the blood, it was possible that normal blood sugars could have occurred in the presence of extensive necrosis of the islets, if the livers were also extensively damaged. However, this was not the case for histological examination of the pancreas showed that the beta cells were normal when adequate doses of sulfhydryl compounds were given before alloxan.[13]

In explaining the protective effect of sulfhydryl groups one should consider the following possibilities:

1. Direct inactivation of alloxan by the SH group of glutathione or cysteine.
2. Protection of the sulfhydryl groups of enzymes from inactivation by alloxan.
3. Reactivation of sulfhydryl groups of

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† In 5 of 10 rats given alloxan i. p. and cysteine i. v. ascites appeared on the 15th-28th day. The rats suddenly began to gain weight rapidly and in some cases the abdominal cavities contained as much as 100 cc of fluid. The livers were shrunken and had a thickened capsule. Further studies are being carried out.

[14] Richardson, G. M., and Cannan, R. K., *Biochem. J.*, 1929, **23**, 68.

[15] Archibald, R. M., *J. Biol. Chem.*, 1945, **158**, 347.

enzymes inactivated by alloxan.

Alloxan can react directly with cysteine to form dialuric acid,[16,15] which is probably not diabetogenic.[17,15] Bruckman and Wertheimer[18] reported that a "super-saturated" solution of dialuric acid injected intravenously into rats did produce diabetes, but, since dialuric acid is rapidly converted to alloxan on standing in air,[15] their results might be due to alloxan contamination.

Ascorbic acid is also reported as capable of reducing alloxan to dialuric acid.[19] If the mechanism of sulfhydryl protection were simply reduction of alloxan to a nondiabetogenic compound, dialuric acid, one would also expect ascorbic acid to protect against alloxan diabetes. Ascorbic acid does not protect against alloxan diabetes, but rather appears to intensify the severity of the diabetes, for the 48-hour blood sugars are considerably higher in the animals given ascorbic acid plus alloxan than are those of the rats given alloxan alone (610 mg per 100 cc compared with 397 mg per 100 cc). However, interactions of ascorbic acid and alloxan within the body may not occur as readily as they do *in vitro*. Since alloxan exerts its diabetogenic effect within the first few minutes of injection,[9,20] the relative rates of interreaction of alloxan with cysteine or with ascorbic acid might influence their protective abilities.

If the administered sulfhydryl groups were able to reactivate the enzymes inactivated by alloxan, then giving the sulfhydryl compound after the administration of alloxan also should protect against diabetes. Leech and Bailey[9] have shown that alloxan disappears from the blood within 2 minutes following its intravenous injection. Gomori and Goldner[20] found that when the vessels to the pancreas were clamped before and for 5 minutes after the injection of alloxan then the beta cells in the clamped portion of the pancreas did not undergo necrosis. This fact indicates that the alloxan had disappeared from the blood during the period of clamping. However, in the present experiments the administration of cysteine one minute after alloxan produced only partial protection, whereas, when it was given after 3 minutes following the alloxan, no significant protection resulted. Therefore, if alloxan does inactivate the SH groups of enzymes, the administered glutathione or cysteine did not reactivate them after 3 minutes had elapsed. In this connection it is interesting that succinic dehydrogenase which has been inactivated by alloxan can be only partially reactivated by glutathione.[5] The action of glutathione and cysteine, therefore, may consist of a protection of the sulfhydryl groups of the islet cells from inactivation as a consequence of the administration of large amounts of exogenous sulfhydryl compounds. Although this protection may simply be due to direct inactivation of alloxan by the administered SH groups, a more complex mechanism may also be involved.

The mechanism by which alloxan produces selective degeneration of the beta cells is not understood. Although other cells (kidney, liver, and occasionally, adrenal) may, at times, show degeneration following alloxan administration, the beta cells of the islets are by far the most sensitive to alloxan. Since the beta cells are highly specialized cells, for their main function apparently consists of the synthesis and secretion of insulin, their enzyme systems are probably also highly specialized. In contrast, the liver and kidney cells which do many things may have a number of alternate enzyme pathways. If the enzyme systems of the highly specialized beta cells require active sulfhydryl groups, whereas the less specialized cells of the body possessed alternate nonsulfhydryl pathways, the apparent selectivity of alloxan for the beta cells might be explained. However, this hypothesis could not easily explain the occasional occurrence of necrosis in the kidney or liver after the administration of

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[16] Labes, R., and Freisburger, H., *Arch. f. exp. Path. u. Pharm.*, 1930, **156**, 226.

[17] Goldner, M. G., and Gomori, G., *Endocrinology*, 1944, **35**, 241.

[18] Brückman, G., and Wertheimer, E., *Nature*, 1945, **155**, 267.

[19] Ruben, J. A., and Tipson, R. S., *Science*, 1945, **101**, 536.

[20] Gomori, G., and Goldner, M. G., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 232.

alloxan.

Following the injection of alloxan into animals, there is a rapid decrease in the glutathione content of the body. DeCaro and Rovida[21] reported that within 10 minutes following the injection of alloxan into rats (200 mg/kg i.p.) the glutathione content of the liver fell to 17% while that of the intestine fell to 42% of their normal values. Leech and Bailey[9] reported that following the injection of alloxan into rabbits (200 mg/kg i.v.) the blood glutathione dropped rapidly in some cases to almost zero. Within 5 minutes after the injection the blood glutathione values began to rise and returned to normal by 18 to 24 hours.

Because injected alloxan causes a decrease in the glutathione content of the tissues, and because glutathione protected against alloxan damage, it is possible that the amount of glutathione present within the cell may determine its resistance to alloxan. If the beta cells of the islets of Langerhans contained less glutathione than other cells of the body, their greater susceptibility to alloxan might thus be explained.

Various species have been reported to have different susceptibilities to alloxan. Hooded rats, for example, are more resistant to alloxan than are albino rats.[22] Whereas a rat becomes diabetic after a dose of 40 mg/kg i.v., human beings can tolerate much larger doses without showing any effect.[23,24] However, a single dose of alloxan (600 mg/kg i.v.) did produce an effect on the blood sugar and islets of humans.[24] Variations in species susceptibility to alloxan might also be explained, in part at least, by differences in the amount of glutathione or cysteine they contain.

The question of whether alloxan may be involved in human diabetes is an interesting one.

1. Theoretically, at least, alloxan could be formed from uric acid, a normal metabolite. Under certain conditions an enzyme present in dog liver can convert uric acid into dialuric acid.[25] Dialuric acid is rapidly oxidized to alloxan by air. This latter reaction, however, may not occur as readily within the body, for the oxidation of dialuric acid to alloxan is inhibited by protein.[26] Studies of the possible formation of alloxan from uric acid within the human body need to be undertaken.

2. The changes seen in the pancreas of rats which have been given alloxan and have been diabetic for many months are not greatly different from those seen in human diabetes. Although some investigators have stated that the cytological changes of the pancreas in alloxan diabetes are quite different from those occurring in human diabetes, they have usually compared the lesions seen in human diabetics with those occurring in the acute stages following alloxan administration.[27] The islets of diabetic rats 5 to 7 months after the injection of alloxan appeared essentially normal when examined with the usual histological stains (hematoxylin and eosin). However, when they were examined by special granule stains, they showed a marked decrease in the number of beta cells present.[28] Similarly, the islets of human diabetics often appear normal when examined by the ordinary histological methods. It is doubtful whether the hyalinization seen in some cases of human diabetes is significant for it is not always present and may even be present in the absence of diabetes.[29] However, in 3 of 5 human diabetics whose islets

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[21] De Caro, L., and Rovida, E., *Boll. Soc. Ital. Biol. Sper.*, 1937, **12**, 611.

[22] Duff, G. L., and Starr, H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 280.

[23] Brunschwig, A., Goldner, M. G., Allen, J. G., and Gomori, G., *J. Am. Med. Assn.*, 1943, **122**, 966.

[24] Brunschwig, A., Allen, J. G., Owens, F. M., and Thornton, T. F., *J. Am. Med. Assn.*, 1944, **124**, 212; Brunschwig, A., and Allen, J. G., *Cancer Res.*, 1944, **4**, 45.

[25] Ascoli, M., and Izar, G. Z., *Z. physiol. Chem.*, 1909, **62**, 347; Preti, L., *Z. physiol. Chem.*, 1909, **62**, 354.

[26] Dixon, M., and Zerfas, L. G., *Biochem. J.*, 1940, **36**, 371.

[27] Duff, G. L., *Am. J. Med. Sci.*, 1945, **210**, 381.

[28] Lazarow, A., and Palay, S. L., unpublished observations.

[29] Warren, S., *The Pathology of Diabetes Mellitus*, 2nd ed., Lea and Febiger, Philadelphia, 1938.

were studied by special granule stains, Gomori[30] reported a decrease in the relative number of beta cells.

3. Human beings do react to alloxan. Although it was first reported that man does not respond to alloxan, Brunschwig and Allen[24] have since reported that large doses of alloxan do produce an effect.

4. Since alloxan occurs in some plants in its half-reduced form, alloxantin,[31] it might be ingested in the diet. This possibility becomes significant in the light of a report by Ruben and Yardumian[32] that oral administration of alloxan causes diabetes.

5. The amount of alloxan actually necessary to produce diabetes is not large. For example, in the rat a small dose of 40 mg/kg produced diabetes consistently and 20 mg produced diabetes at times. Since the weight of the pancreas is less than 1% of the body weight, and since the beta cells constitute but a small fraction of the weight of the pancreas, the actual amount of alloxan necessary to destroy most of the beta cells in the rat is probably much less than 10 γ.

Glutathione, normally present in the blood and tissues, may serve to protect the body from any alloxan which might appear metabolically or in the diet. However, critical amounts of alloxan sufficient to injure the beta cells might conceivably appear within the human body under a number of circumstances:

a. A metabolic defect resulting in excessive accumulation of alloxan in the body.

b. Decrease in glutathione concentration in the body resulting in a decrease in the normal protective mechanism against alloxan.

c. A specific metabolic defect of glutathione occurring only within the beta cells of the islets.

From the evidence at hand it would be desirable to study the relation of alloxan and glutathione metabolism to the etiology of human diabetes.

Summary. 1. The intravenous injection of large doses of glutathione or cysteine, compounds which normally occur in the body, one to 2 minutes prior to the injection of a diabetogenic dose of alloxan completely protected rats from diabetes.

2. Other amino acids, phosphate buffer, and ascorbic acid did not exert such a protective effect.

3. When the sulfhydryl compounds were given one minute after the alloxan injection, partial protection occurred, whereas, when 3 or more minutes had elapsed, there was no protection.

4. The mechanism and significance of this protection are discussed.

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[30] Gomori, G., *Am. J. Path.*, 1941, **17**, 395.

[31] Lippmann, E. O., *Ber.*, 1896, **29**, 2645; Ritthausen, H., *Ber.*, 1896, **29**, 894, 2106; Fisher, H. J., and Johnson, T. B., *J. Am. Chem. Soc.*, 1932, **54**, 2038.

[32] Ruben, J. A., and Yardumian, K., *Am. J. Clin. Path.*, 1945, **15**, 230.

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